Practical Virology

(I)

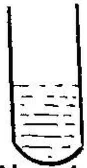
Virus purification and concentration



Viral purification and Concentration

• Virus identification:

1. Serological tests (Ag - AB reaction)
2. Molecular techniques (N.A detection)



Virus that may be

- Low Concentration → False -ve result → So, Concentration of Virus
- with Contaminants (debris) that Leads to misleading results → So, purification of Virus

Virus purification

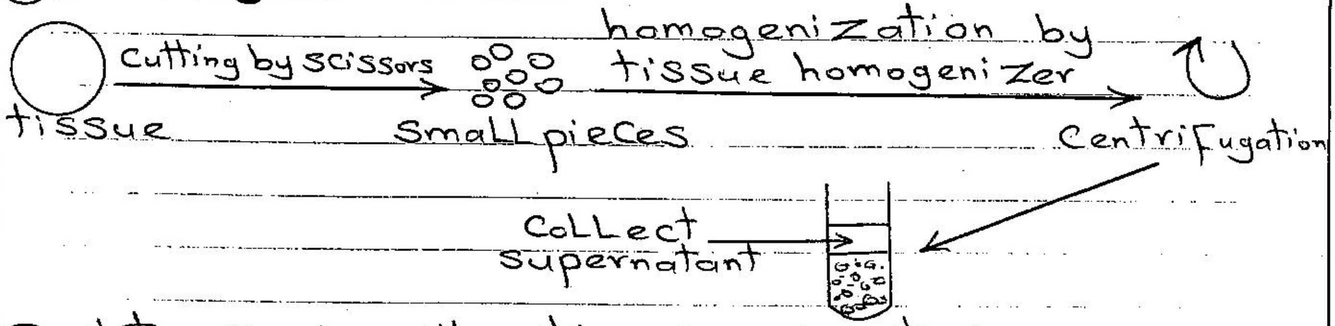
aim of virus purification:

1. Removal of impurities
2. Sample Concentration
3. Morphological study of Virus by EM.
4. Chemical studies of virus
5. preparation of Vaccine
6. preparation of antisera

How to release Virus from Cell?

Liberation of virus from cells by:

① Homogenization:



② ultrasonic vibration (Sonicator)

③ Repeated freezing and thawing

Methods of purification:

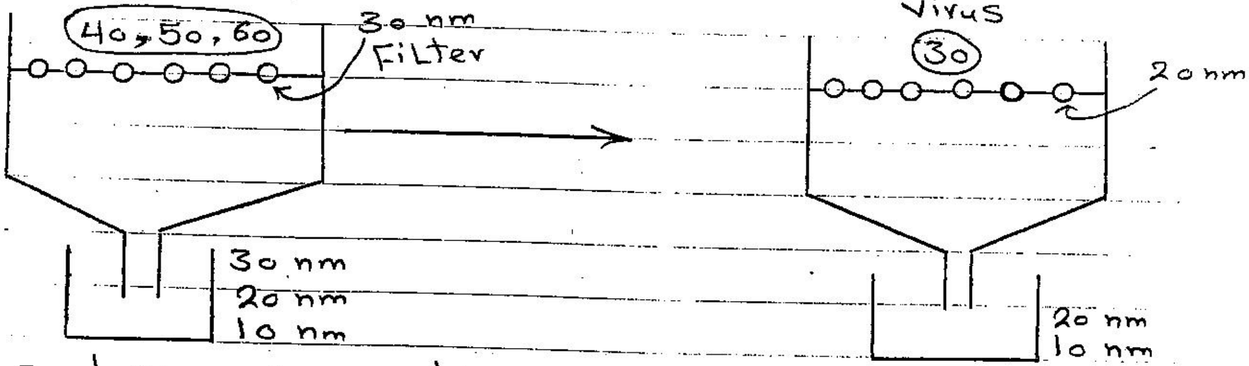
physical methods	chemical methods	Biological methods
1_ultrafiltration	1_precipitation of virus	1_Haemagglutination and elution
2_ultracentrifugation	2_extraction with solvents	2_Virus neutralization
3_Ion exchange chromatography	3_2 phases aquous polymer method	3_digestion with enzymes
4-gel filtration	4-formation of complex compound.	
5_electrophoresis		
6_Dialysis		

(I) physical methods

depend on size	depend on charge	depend on size and charge
1_ultrafiltration	Ion exchange chromatography	electrophoresis
2_ultracentrifugation		
3-gel filtration		

1) Ultrafiltration:

- depend on virus size
- by using → Collodion filter
  → Nitrocellulose membrane
- under pressure
- Large particles not penetrate and small particles pass



- used for separation of viruses of different size → so, virus of large size trapped (retained) and vice versa.

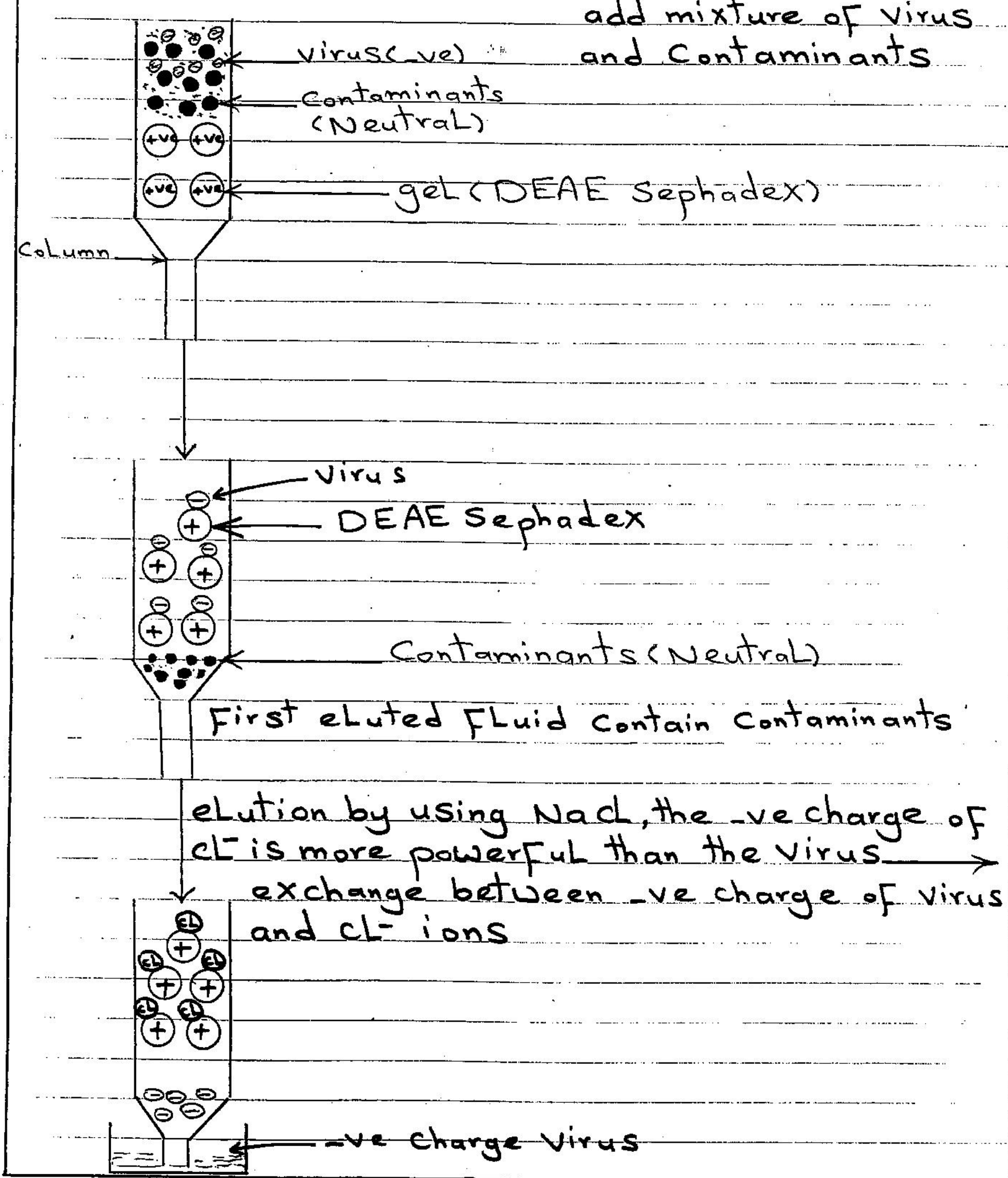
2) Ion exchange chromatography:

Viruses acc. to its charge:

+ve charge virus → gel matrix (-ve) charge
Diethyl aminoethyl Cellulose (DEAE Cellulose)

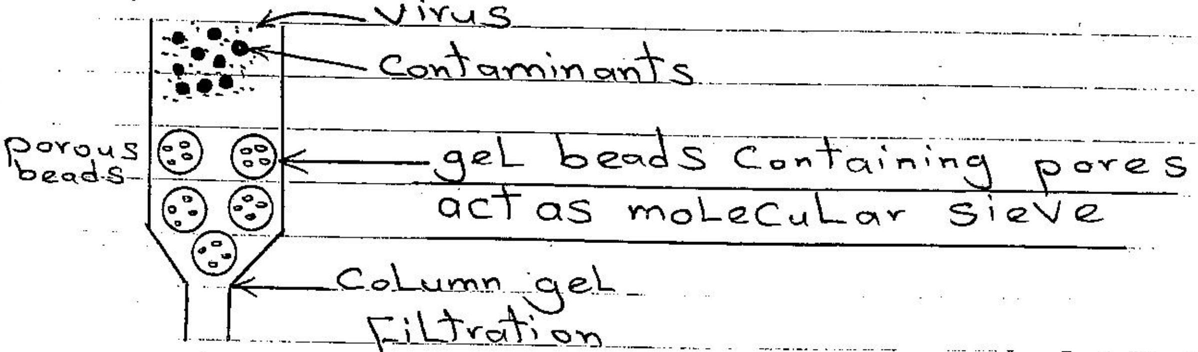
-ve charge virus → gel matrix (+ve) charge
Diethyl amino ethyl Sephadex (DEAE Sephadex)

- depend on charge of virus.

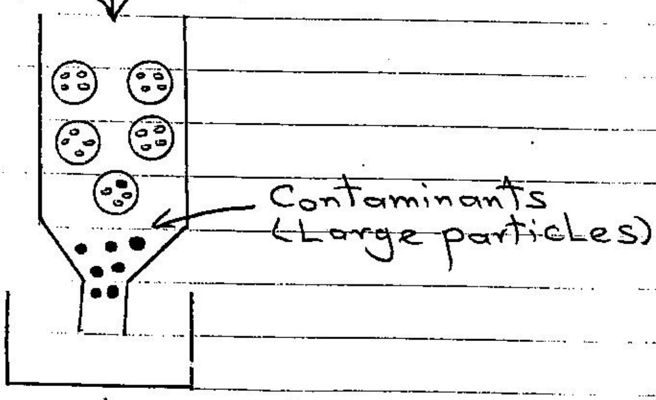

add mixture of Virus and Contaminants
Virus(-ve)
Contaminants (Neutral)
+ve
gel (DEAE Sephadex)
Column
Virus
DEAE Sephadex
Contaminants (Neutral)
First eluted fluid contain contaminants
elution by using NaCl, the -ve charge of Cl⁻ is more powerful than the virus → exchange between -ve charge of virus and Cl⁻ ions
Cl
-ve charge Virus

③ gel Filtration:

- depend on virus size

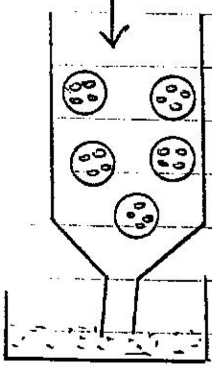


Virus enter beads



the First eluted Fluid Contain Contaminants

Washing



the second eluted Fluid Contains virus (virus eluted Later)

④ geL eLectrophoresis:

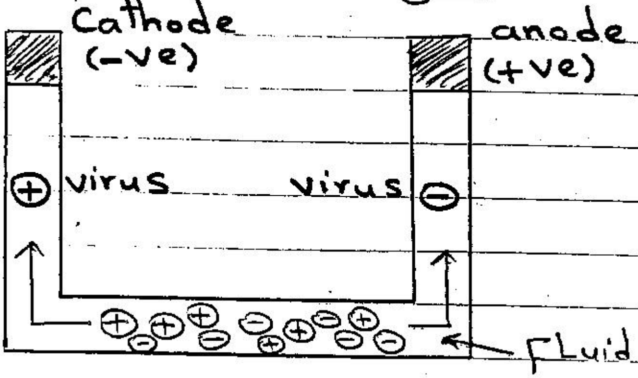
depend on Charge and SiZe of Virus

- Moving of Charged moLeCuLes in eLectric FieLd

Types of geL eLectrophoresis:

Moving boundries eLectrophoresis

depend on charge

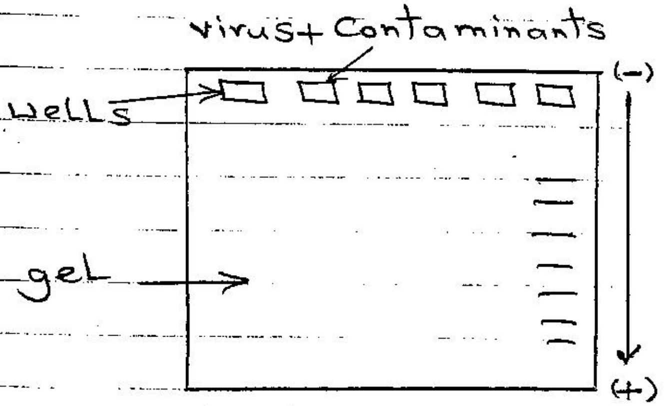


+ve Virus ⟶ migrate to Cathode

-ve Virus ⟶ migrate to anode

Zone geL eLectrophoresis

depend on Charge and size



at pH 8, Virus is -ve

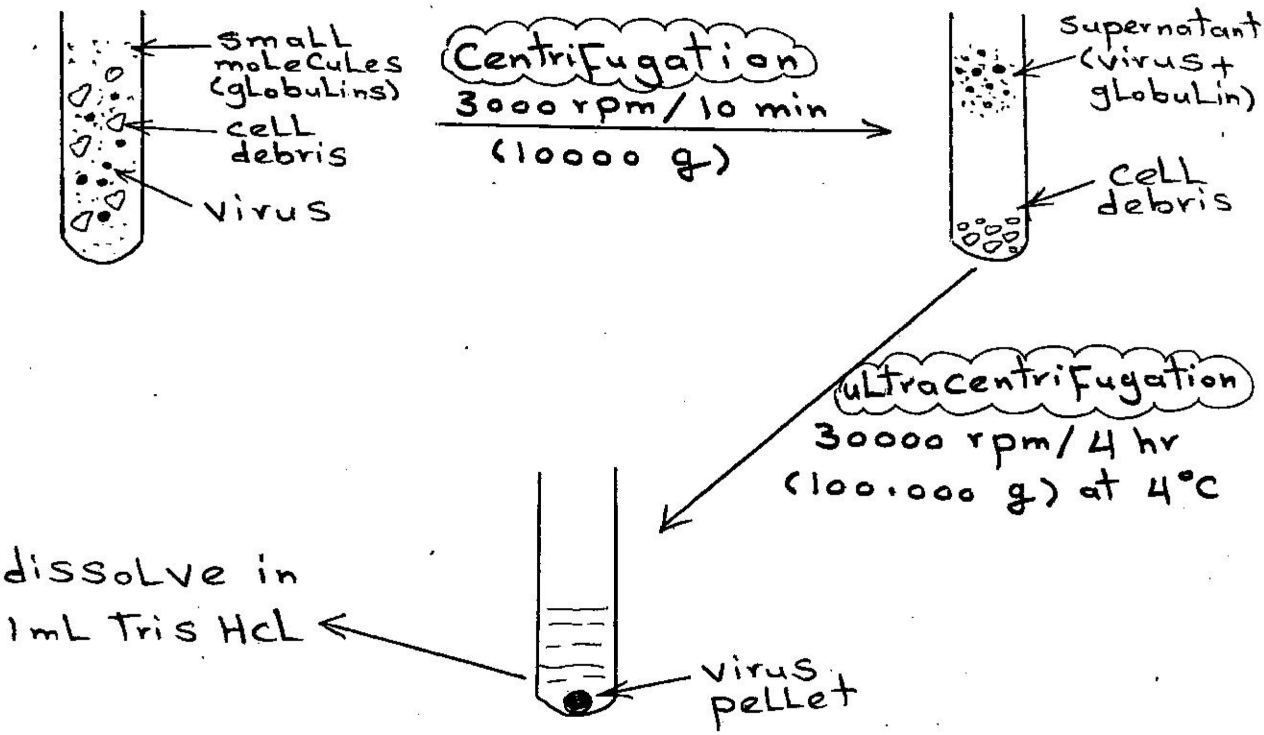
→ smaLLer virus migrate faster than Large one.

⑤ ultracentrifugation:

- differential ultracentrifugation
- density gradient
  - Rate Zonal
  - equilibrium density gradient ultracentrifugation

a- differential ultracentrifugation

def. alteration between centrifugation (Low speed) and ultracentrifugation (high speed)



b- density gradient ultracentrifugation

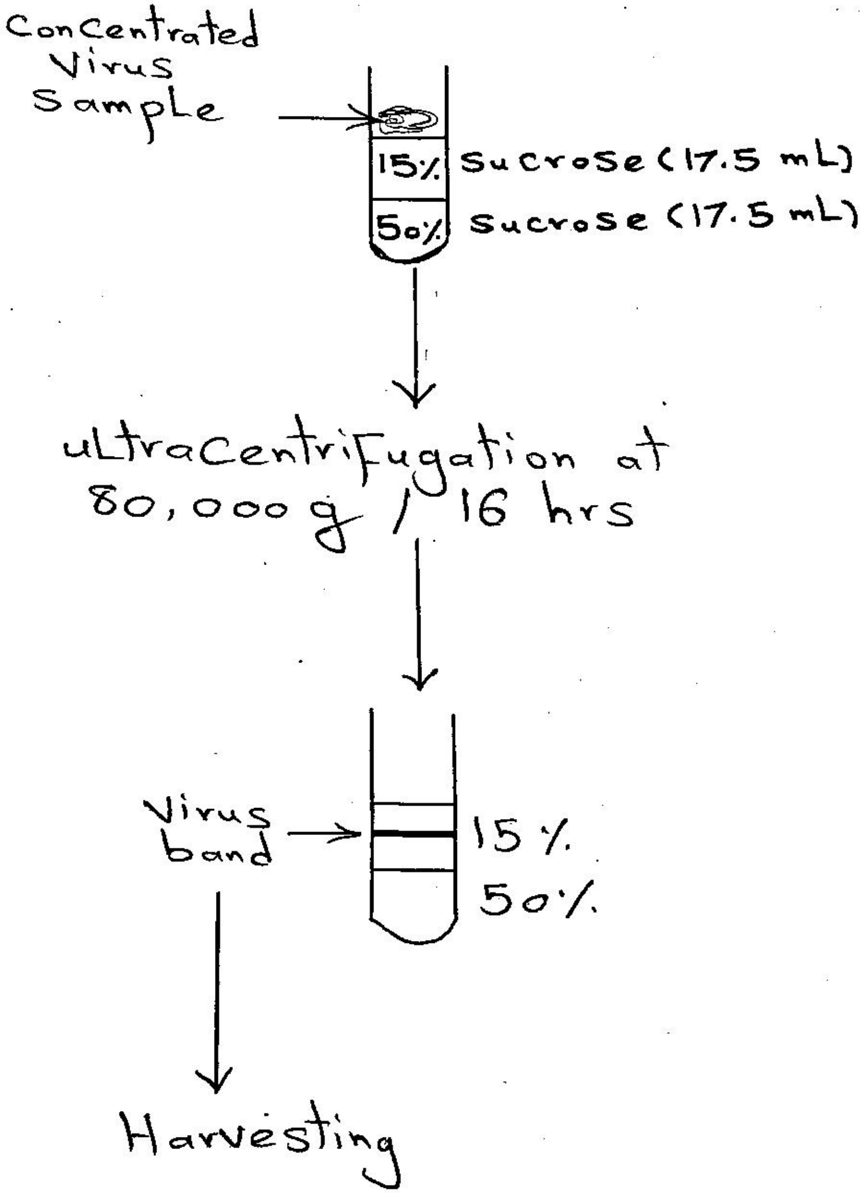
Rate Zonal	Equilibrium density gradient (Isopycnic)
gradient from sucrose or glucose	gradient from Caesium Chloride
depend on sedimentation rate (size)	depend on buyont density
differentiate between viruses differ in size and have the same density.	differentiate between viruses differ in density and have the same size
For enveloped viruses	For naked viruses

Equilibrium density gradient

1- equilibrium density gradient purification on sucrose gradient.
2- equilibrium density gradient purification on Caesium Chloride gradient
3- velocity gradient purification

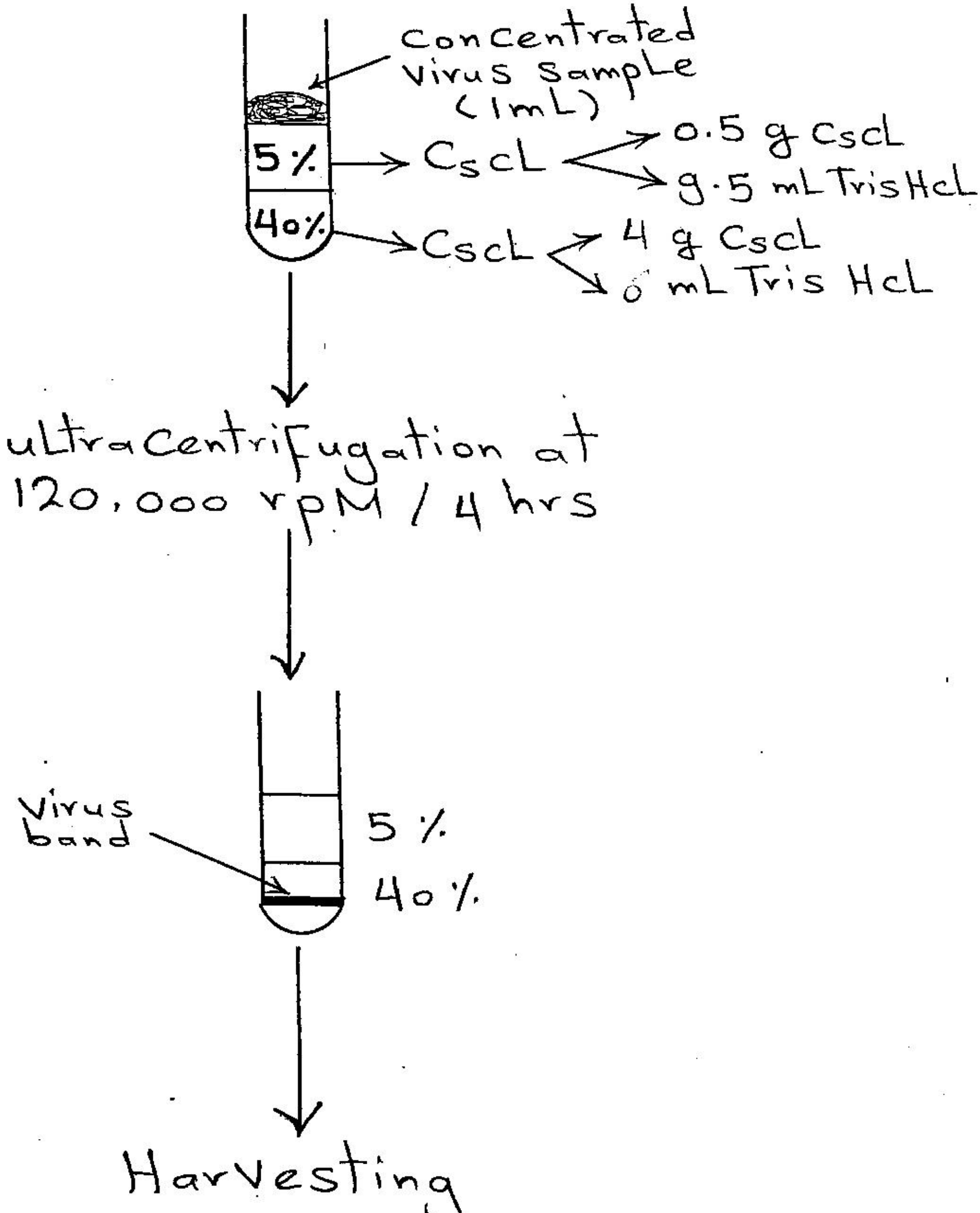
1- Equilibrium density gradient purification on sucrose gradient:

→ For Retroviridae (avian Leukosis)



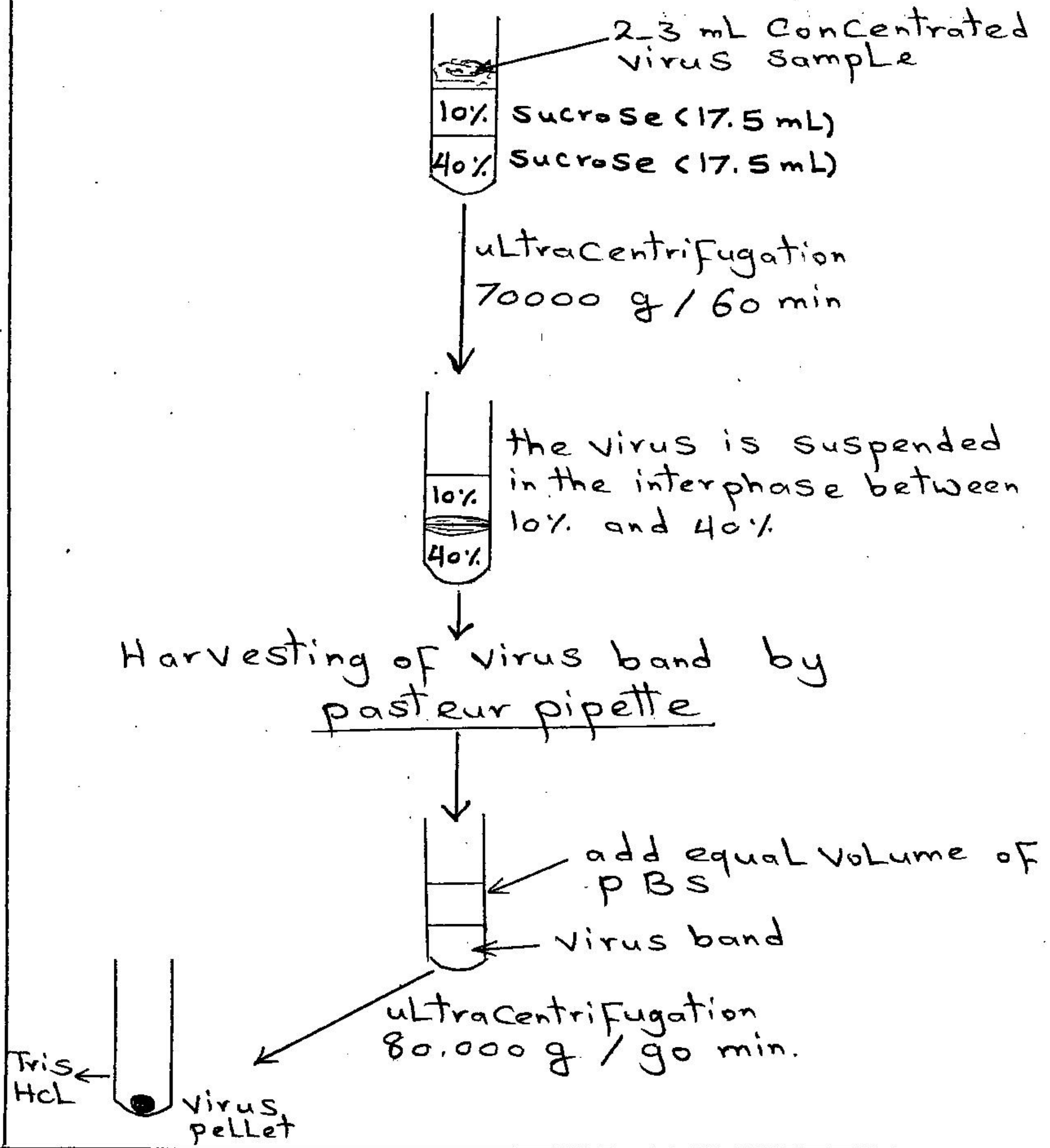
2- Equilibrium density ultracentrifugation on Caesium chloride gradient:

→ For picornaviridae (FMDV - poliovirus)



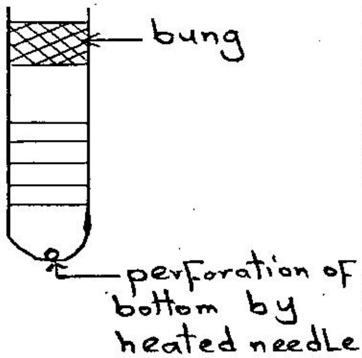
3- Velocity gradient purification (sucrose)

→ orthomyxoviridae (Influenza Virus)



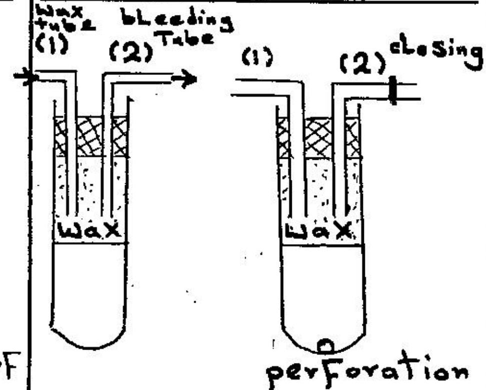
Harvesting of virus band from gradient:

by bottom puncture into estimated Fraction



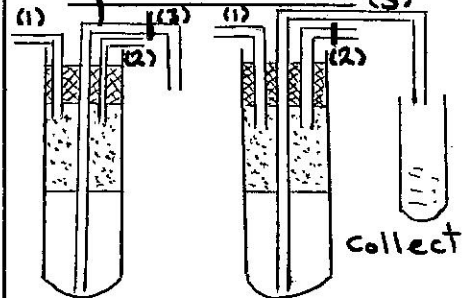
1. insertion of bung
2. performation of bottom by heated needle
3. appliCation of pressure on bung.
4. harvesting of virus band (gradient) in collecting vessels

by bottom puncture into Known volume



1. pouring of wax from tube(1) till release from tube(2) (bleeding tube)
2. closing of tube(2) by clips
3. perforation of bottom by heated needle.
4. addition of Known volume of wax from tube(1) Lead to release of the same volume of the gradient from bottom.

displacement without bottom puncture



1. close tube(3) by clips.
2. pouring of wax from tube(1) till release from tube(2)
3. closing tube(2) (bleeding tube) by clips.
4. opening tube(3)
5. pouring of wax from tube(1) Lead to release of the same volume of the gradient from tube(3) by displacement.

Q: Methods of virus purification depend on size:

1- gel filtration
2- ultrafiltration
3- gel electrophoresis ⟶ Zone gel electrophoresis.
4- Rate zonal density gradient ultra-centrifugation.

Q: Methods of virus purification depend on charge:

1- gel electrophoresis (Moving boundaries)
2- Ion exchange Chromatography

Q: Method of virus purification depend on size and charge:

Zone gel electrophoresis

Q: Method of virus purification depend on density:

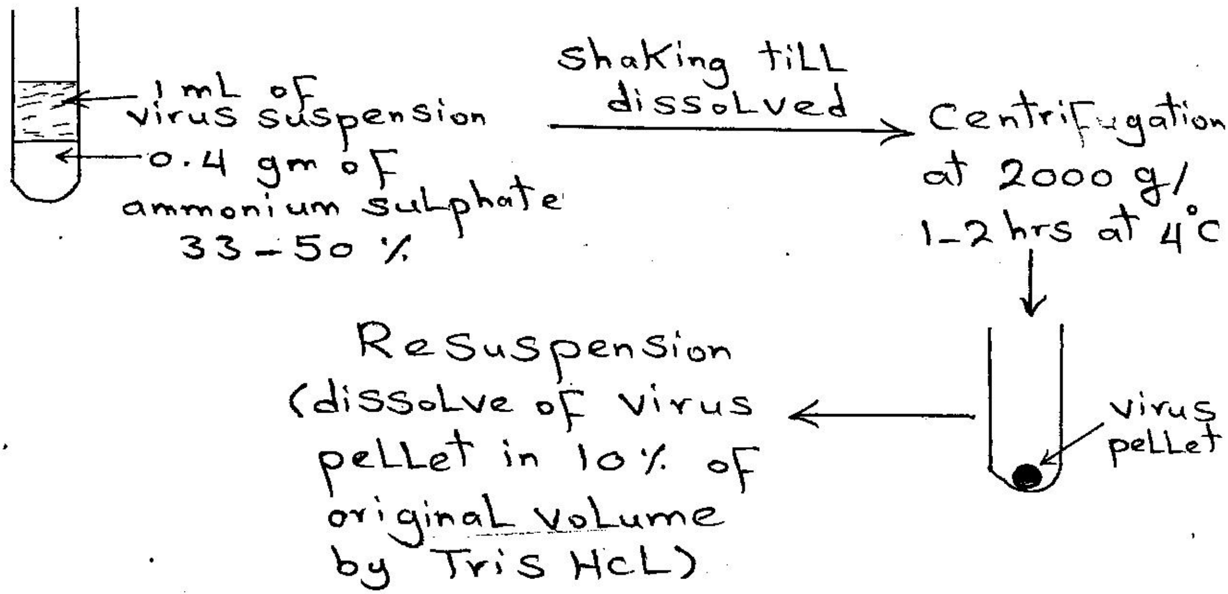
Equilibrium density gradient ultracentrifugation (Isopycnic)

II Chemical methods

1 precipitation:

a- with ammonium sulphate:

For Tobacco mosaic virus (TMV)



1000 mL of virus → Concentrated to → 100 mL

b- Cold alcohol (0-2 °C):

→ ethanol (25-35%) ⟶ Influenza virus

→ Methanol (0.5 %) ⟶ poliovirus

- Cold alcohol (0-2°C) is used to avoid denaturation of viral protein.

C-polyethylene glycol (pEG) 5%

→ For paramyxoviridae (Mumps virus, NDV)

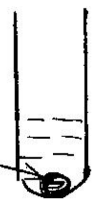


100 mL of Virus suspension

5 g pEG

shaking for 4-5hrs at 2-4 °C → then Centrifugation at 1600 rpm/ 16 hrs

↓



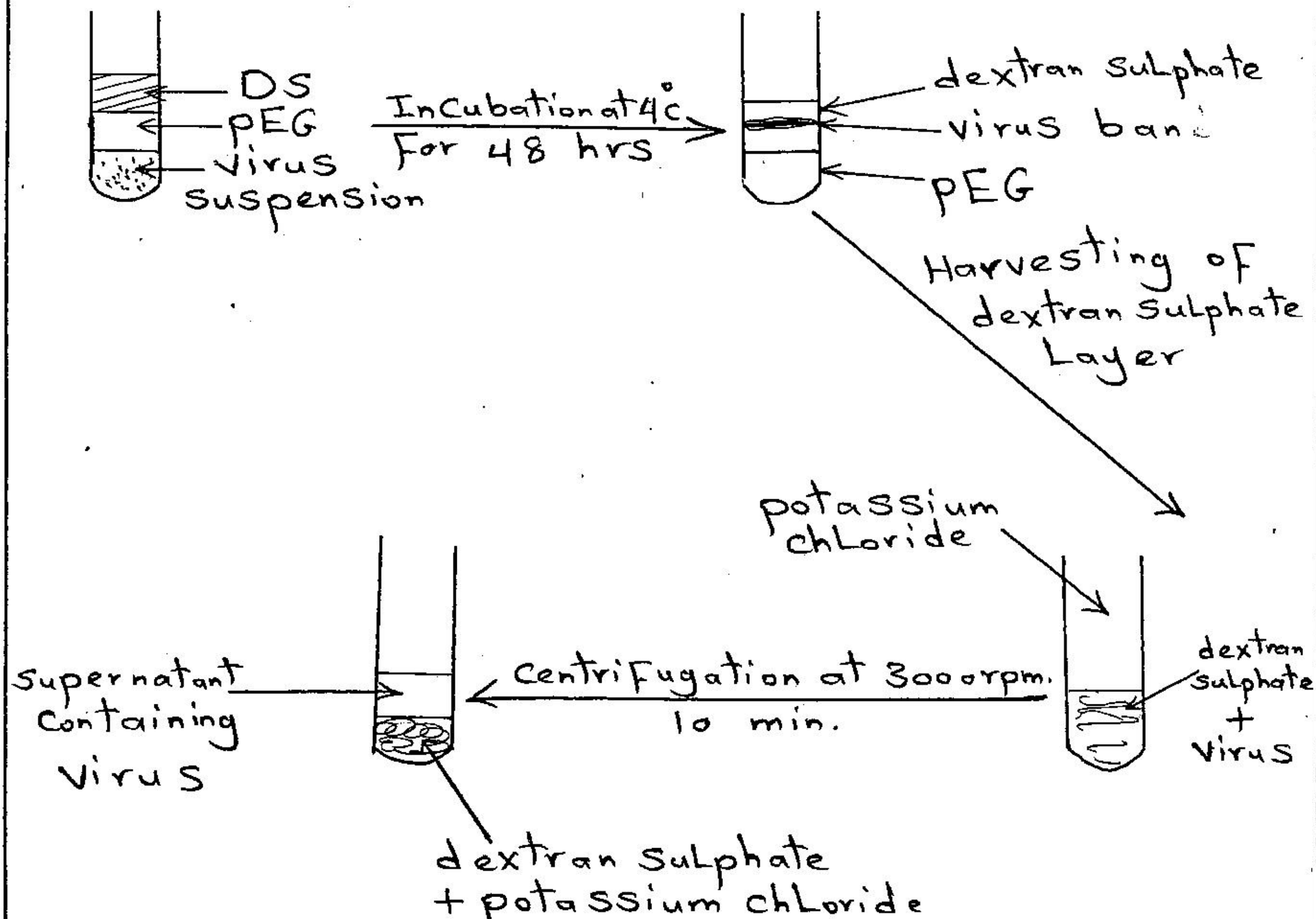
Virus pellet → Resuspension of virus pellet (1mL of Tris HCL for each 100 mL of original Volume = 1% of original Volume)

1000 mL of virus Concentrated to → 10 mL

② 2 phases aquous polymer method:

2 solvents → polyethylene glycol (pEG)
→ dextran sulphate

→ used for FMDV, NDV, polio V, adeno V



3 extraction with solvent:

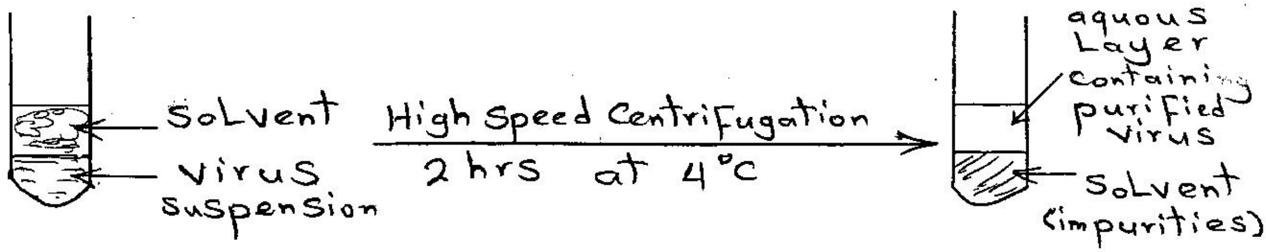
using organic solvents which dissolve cellular protein without denaturation of viral protein as:

1- Arcton 113 → For enveloped viruses as NDV, VSV

2- Sodium dodecyl sulphate ← For naked viruses as FMDV

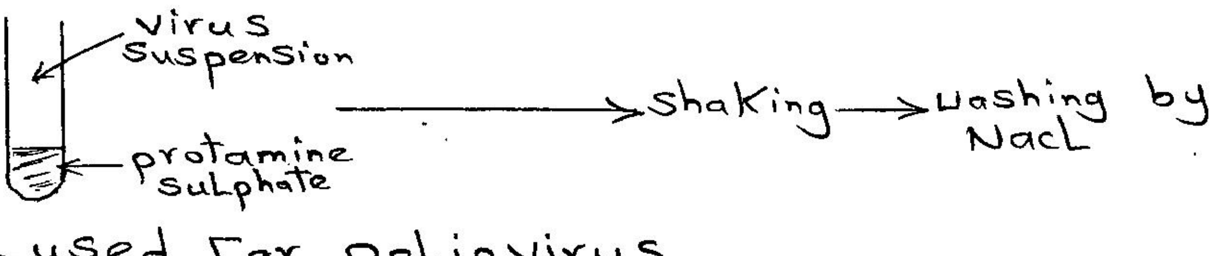
3- Sodium deoxy cholate ←

4- Dichloro-difloroethane → For vaccinia virus



4 Formation of Complex Compound:

binding of acidic group of virus with strong basic protein (protamine sulphate) → Formation of complex → exchange of acidic group of virus with high acidic salt soln. (NaCl)



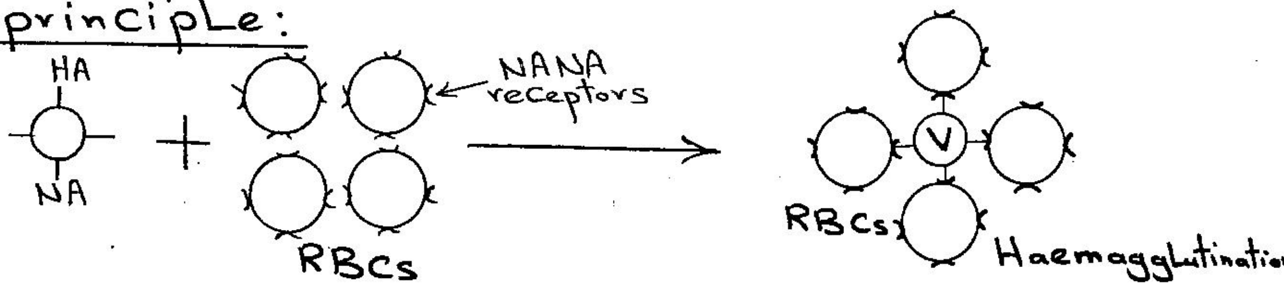
→ used for poliovirus.

III Biological methods

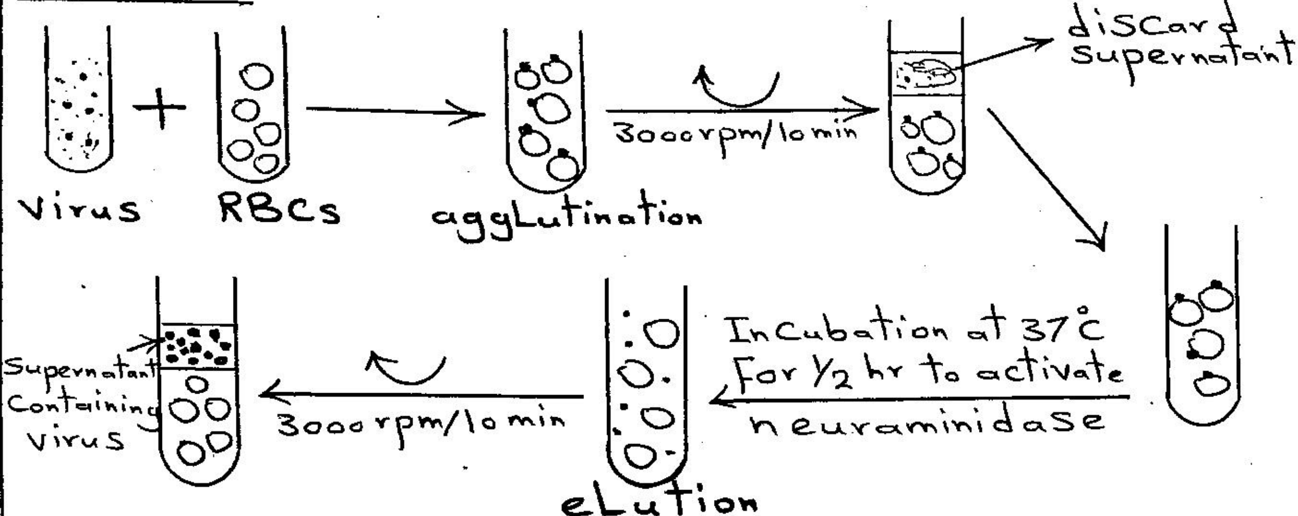
1) Haemagglutination and elution:

- depend on structure of Viral envelop (HA, NA)
- used for <u>enveloped viruses</u> (orthomyxoviruses and paramyxoviruses).

<u>principle:</u>



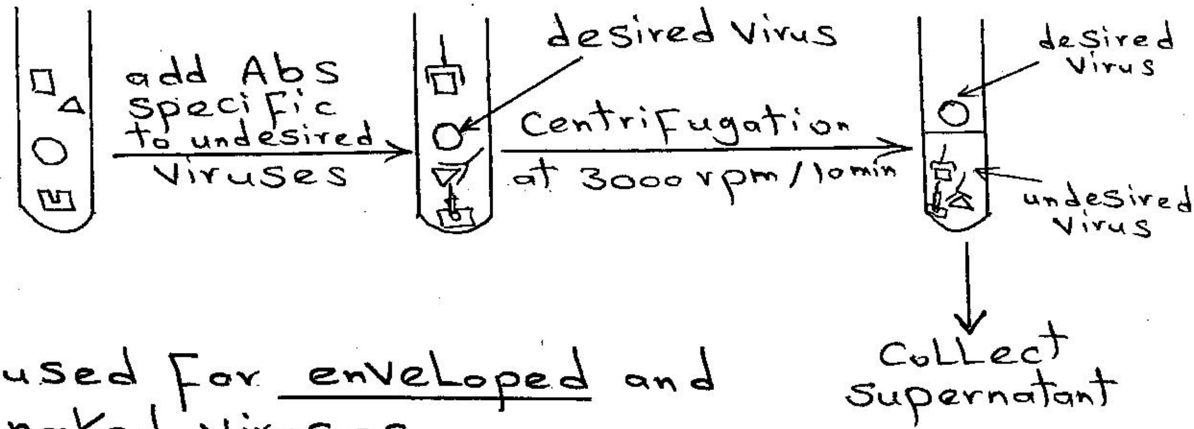
- <u>at 37°c</u> → Neuraminidase (receptor destroying enzyme) → breakdown of NANA receptors →
<u>Elution</u>



(due to activation of neuraminidase that breakdown NANA receptors)

② Virus Neutralization:

purification of desired virus from undesired viruses in case of <u>mixed infection</u>.



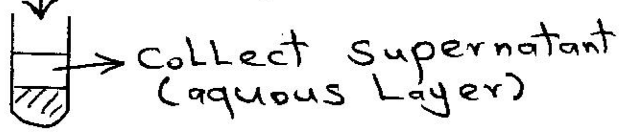
→ used for <u>enveloped</u> and <u>naked</u> viruses.

③ Enzymatic digestion:

digestion by

- Trypsin enzyme → to digest protein contaminants
- Nuclease enzyme → to digest nucleic acid contaminants

↓ Centrifugation



→ <u>used for enveloped viruses as NDV</u> because it is surrounded by lipid bilayer (not affected by Trypsin & Nuclease

Virus Concentration

Small volume

- **ultracentrifugation**
  For orthomyxoviridae (Influenza virus)
- **precipitation**
  - ammonium sulphate → picornaviridae
    - → poliovirus
    - → FMDV
  - polyethylene glycol (pEG) → paramyxoviridae (NDV and Mumps virus)

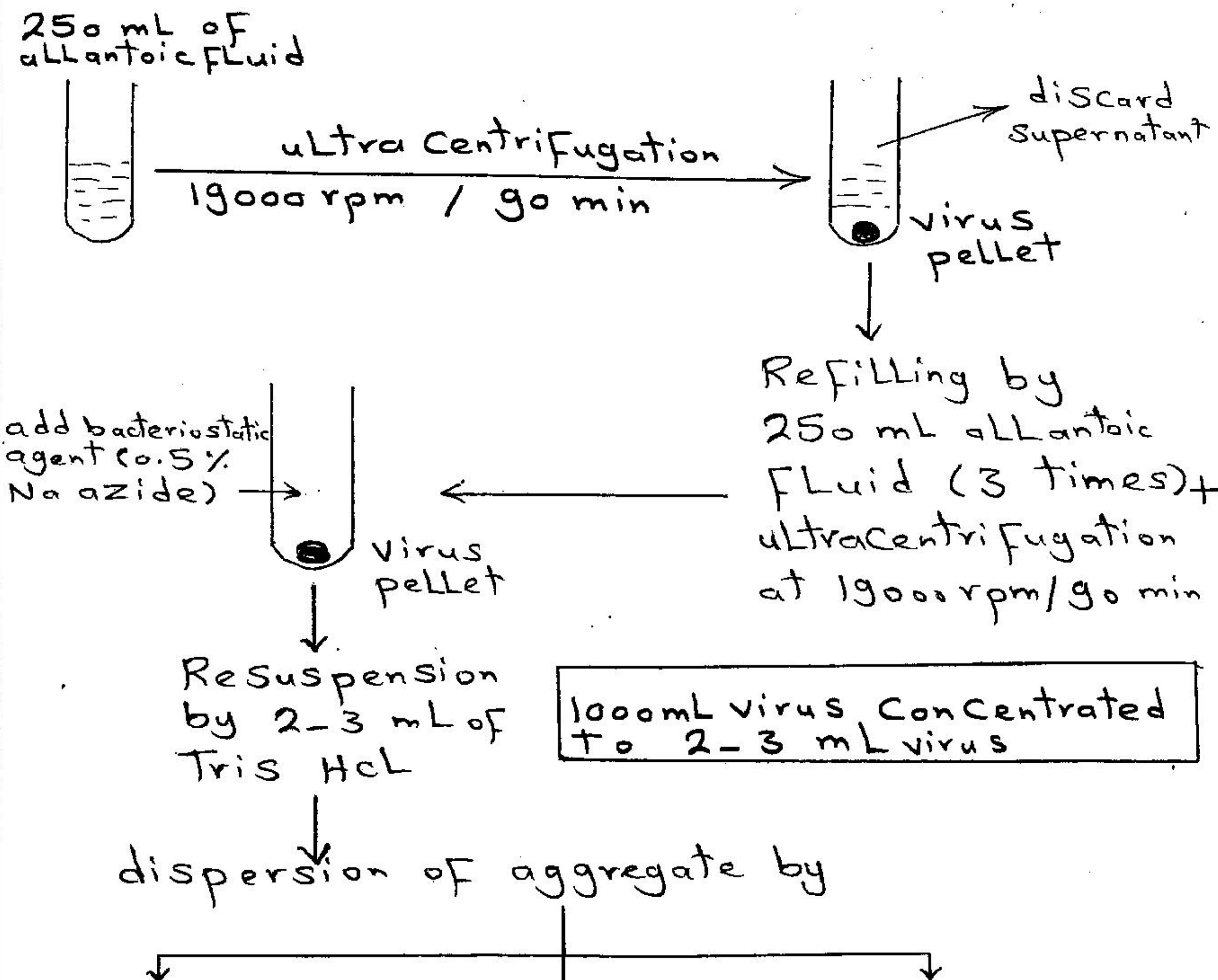
Large volume

1- ultracentrifugation
2- precipitation
3- dialysis

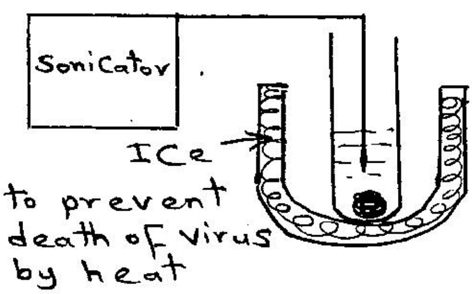
ultracentrifugation

→ mainly used for Influenza virus.
→ Isolated in allantoic cavity of ECE
→ each egg → 10 mL allantoic fluid
100 eggs → 1000 mL allantoic fluid →

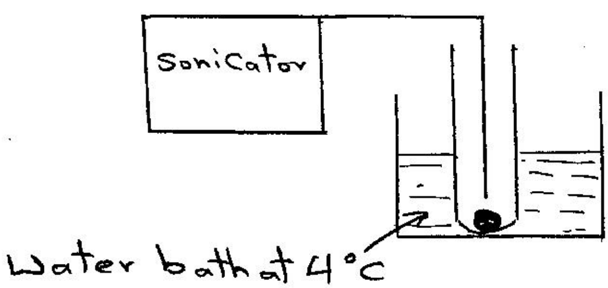
each mL contain 0.5 - 25 mg virus depending on growth activity of virus.



Sonicating probe

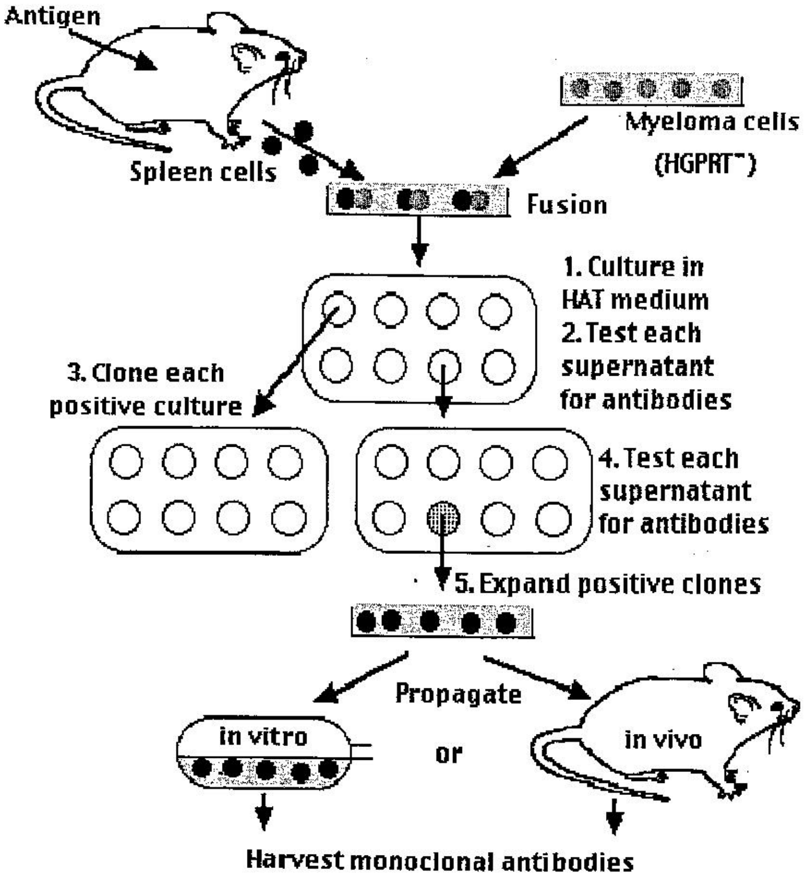


Sonicating water bath

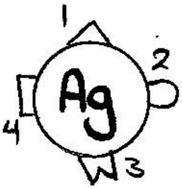


Practical Virology (2)

Serological Tests



polyclonal and monoclonal antibodies

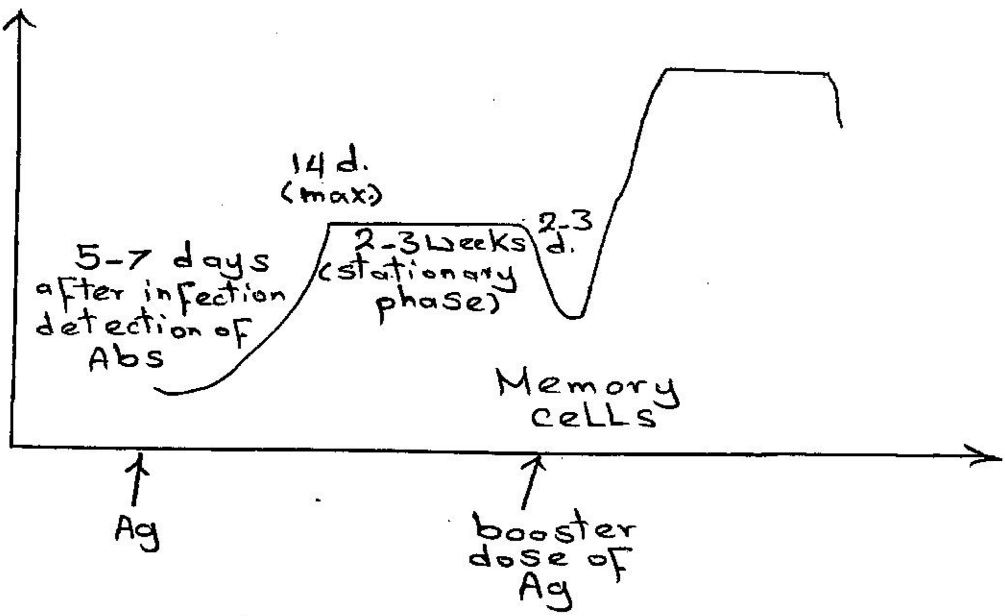


→ each epitope stimulate B_cell proliferate → B_cell clones → plasma cells → Abs complementary to epitope → called **polyclonal antibodies (polyclonal antisera)**

polyclonal antibodies:

antibodies against different epitopes of the antigen produced from different B_cell clones.

antibody production:



polyclonal antibodies production:

mainly in Rabbit.

day 1
- → Collect 15 mL blood from ear vein (-ve serum control)
- → Virus inoculation (1 mL)
  - → 0.5 mL of virus + 0.5 mL of Freund's adjuvant (delay Ag release)
  - → S/C inoculation at 4 different sites, 0.25 mL (Neck and Flank)

↓

day 10 ← Collect serum and compare Abs titre with -ve serum control

↓

day 28 → virus inoculation

↓

day 38 ← Collect serum and compare

↓

day 56 → Virus inoculation

↓

day 66 ← Collect serum and compare

↓

day 84 → virus inoculation

↓

day 94 ←

↓

till day **182** → virus inoculation

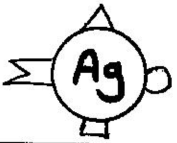
حقن كل ٢٨ يوم
وبعد ١٠ أيام من
كل حقنة نأخذ
عينة serum
ونقارنها بـ control
عشان نشوف نسبة
الـ Abs

after 10 days, exsanguination of animal and collect serum (polyclonal antibodies)

disadvantages of polyclonal Abs:

1. Low Concentration
2. Low specificity → cross reaction → because 2 antigens may share one or more epitopes

Monoclonal antibodies (MAbs)



→ Mice → Serum (polyclonal Abs)
→ Spleen (taken after 21 days)

↓

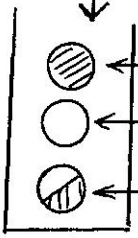
plasma cells

Myloma cells (malignant tumor of Ab producing plasma cells)

Fusion

PEG (polyethylene glycol) (Fusing agent)

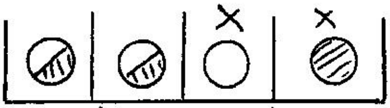
↓



← plasma cells
← Myloma cells
← hybridoma cells

↓

Culturing on HAT media in microdilution tray (96 well plate) (each 50 micron contain one cell)



↓ incubation for 14 days

characters of plasma cells:

1. Specific Ab production
2. grow on HAT media due to presence of HPRT enzyme (HAT resistant)
3. Short life span (2 - 3 weeks)

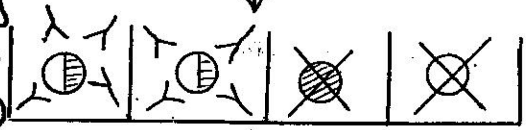
characters of Myloma cells:

1. Non-Specific Ab production
2. Not grow on HAT media due to no HPRT enzyme (HAT Sensitive)
3. Immortal

characters of hybridoma cells:

1. Specific Ab production
2. grow on HAT media (HAT resistant)
3. Immortal
4. Myloma + plasma cells in presence of PEG (Fusing agent)
5. used for mass production of specific Abs (MAbs)

Microdilution tray (each 50 micron contain one cell)



production of monoclonal Abs

↓

Testing for desired Abs (by Known Ag) using
- → ELISA
- → RIA (Radio immuno assay)
- → western blotting

↓

Mass production

In Vivo

I/p injection in mice

↓

The cell multiply and produce Large quantities of Abs in ascitic fluid

↓

Collect Ascitic Fluid

In Vitro

↓

by Culturing of hybridoma cells on cell culture (Continuous cell Line) in bottles

HAT medium:

Media Containing:
→ hypoxanthine
→ aminopetrin (inhibit metabolism)
→ Thymidine

① **plasma cell** → Can survive on HAT medium because they have an enzyme called HPRT (hypoxanthine phosphoribosyl transferase) → which help them to utilize hypoxanthine and survive for 1-2 weeks, after that they die.

② **Myeloma cells** → die in HAT medium due to no HPRT enzyme.

③ **Hybridoma cells** → survive and grow on HAT medium.

• uses of monoclonal antibodies (MAbs):

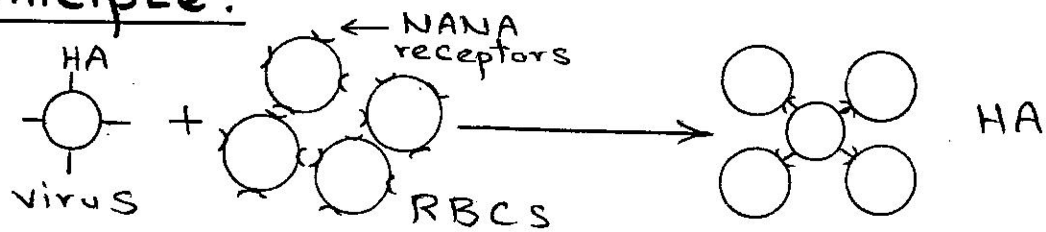
① Immune diagnosis without cross reaction
② Virus purification (by virus neutralization)
③ Virus/Cancer treatment (Magnet bullet)

polyclonal Abs	Monoclonal Abs (MAbs)
antibodies against different epitopes of the antigen produced by different B-cell clones.	antibodies against single epitope of Ag produced by single clone of hybridoma cells.

Haemagglutination test (HA)

def. aggregation of RBCs by virus
(ability of the virus to agglutinate RBCs)

principle:



→ NANA receptors on RBCs surface attach to HA spikes on virus surface

Haemagglutinating viruses:

- → orthomyxoviridae → AIV
- → paramyxoviridae → NDV
- → Coronavirus
- → Togavirus
- → Reovirus
- → enterovirus
- → adenovirus
- → pox virus

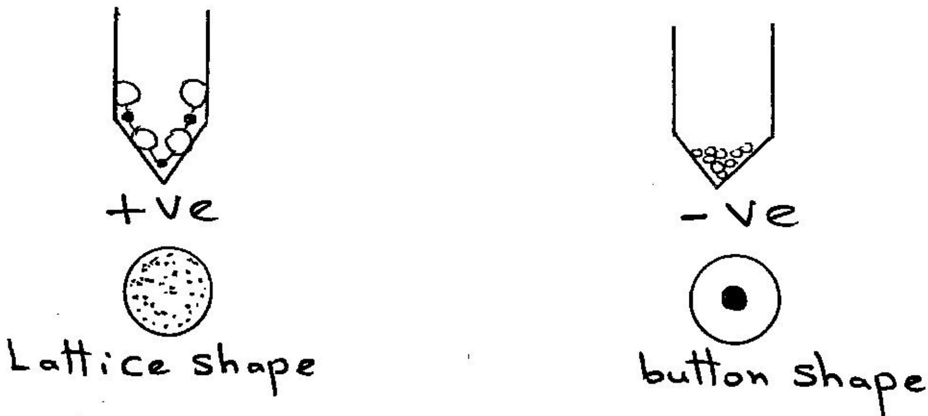
(Reovirus, enterovirus, adenovirus ← Naked viruses)

uses of HA in Virology Lab.:

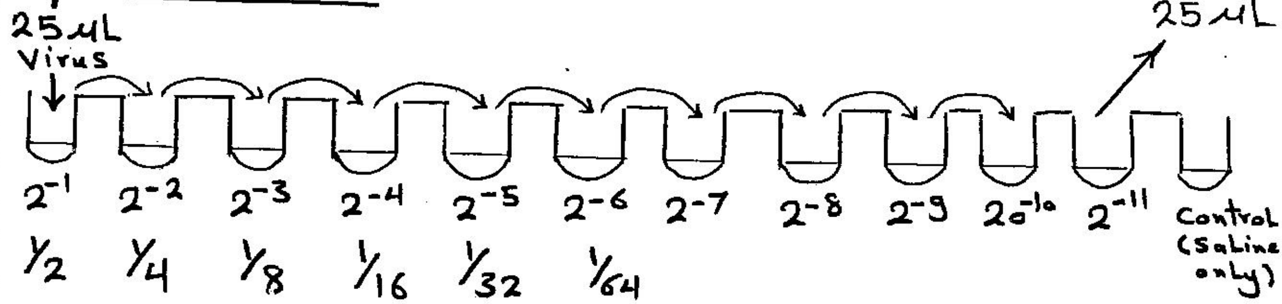
1. detection of virus in TC or clinical specimen
2. virus counting
3. virus purification
4. detection of non-cytocidal viruses
5. preliminary test before HI to determine 4 HAU to avoid excess of virus after virus-antibody reaction.

Haemagglutination test (HA):

- using Microtitre plate with u or V-shaped bottom (96 wells)



- **procedure:**



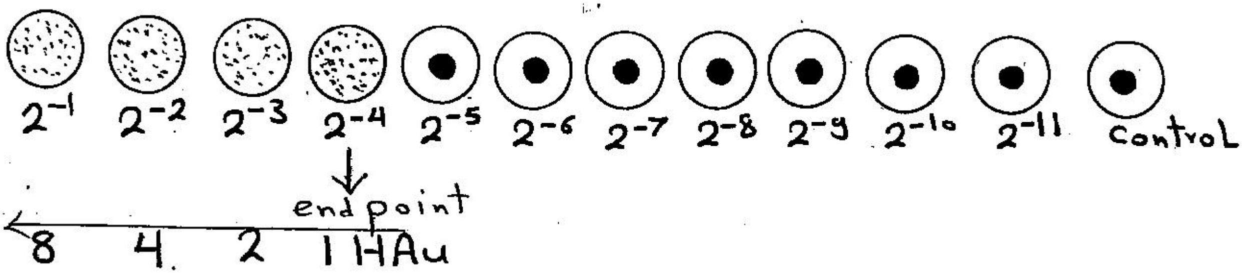
25 µL Saline ——— all wells ———>

25 µL Virus in first well and mix ——> then add 25 µL of mixture to 2nd well and so on ——> before the last well, discard 25 µL ——> the last well contain 25 µL saline only (Control).

25 µL RBCs (0.5 %) ——— all wells ———>

Incubation at 37°C for 1 hr.

Result:

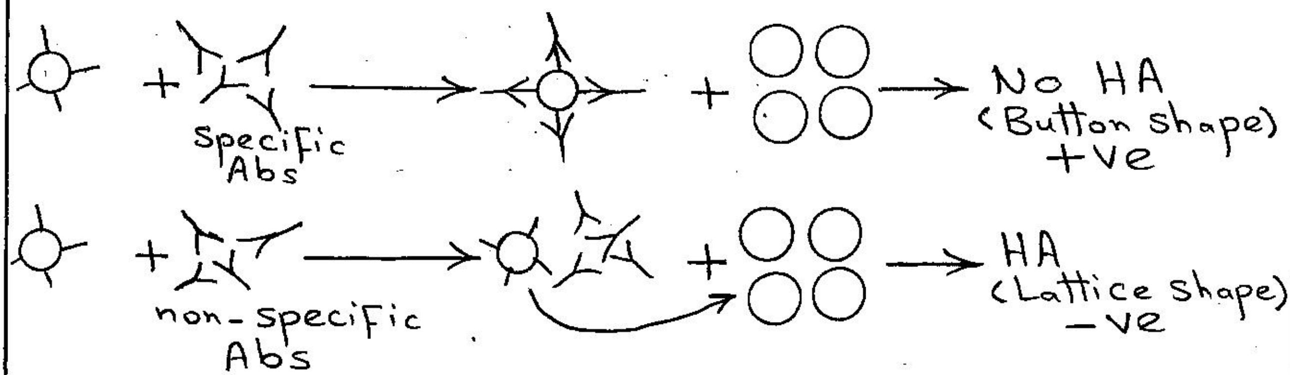


- **End point** → highest dilution of virus causing Complete haemagglutination → $1/16$ (2^{-4})
- **Titer** → reciprocal of end point → 16 (2^4)
- **End point Contain 1 HAu** → so dilution Contain 4 HAu → 2^{-2}

Haemagglutination inhibition (HI)

def. Inhibition of HA by specific antibodies

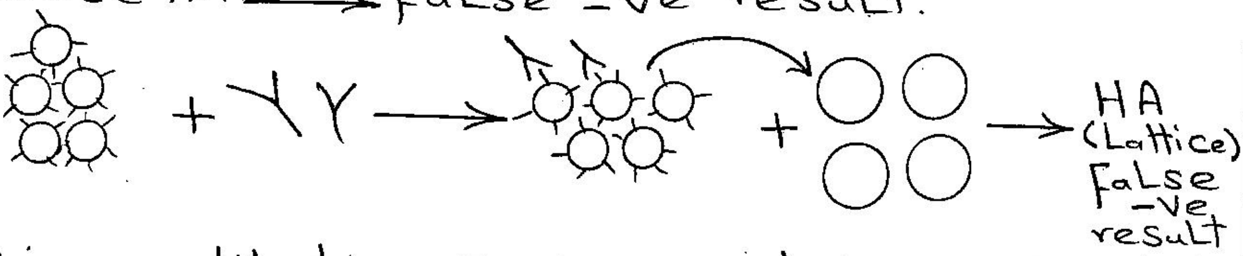
principle:



- Inhibition of haemagglutination of RBCs by haemagglutinating viruses by using Specific Abs

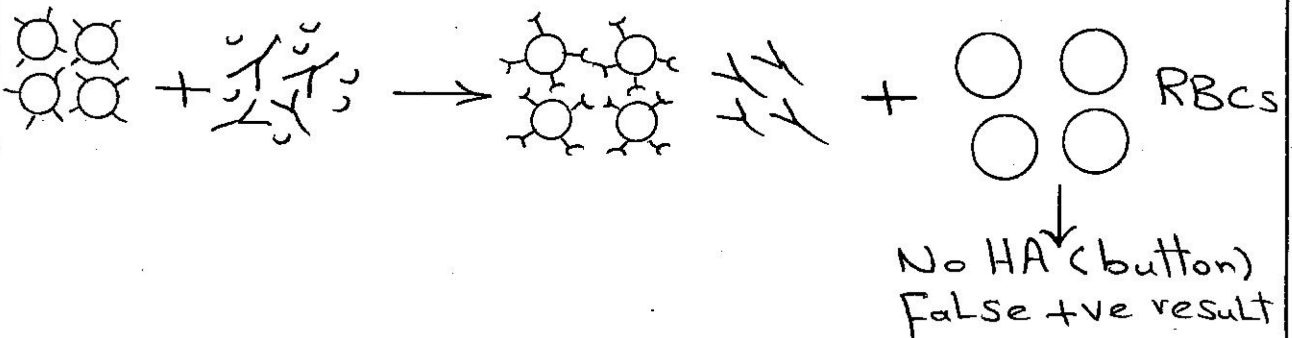
• 2 problems:

① Concentration of virus more than Conc. of Abs → So, some viruses will not bind to Abs and cause HA → False -ve result.



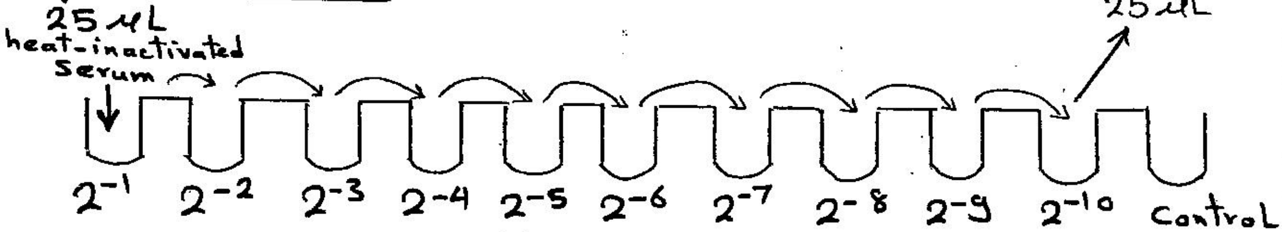
So, use dilution of virus contain 4 HAu that determined by HA test.

② Many sera contain non-specific inhibitors (that act as NANA receptors) → give false +ve result.



So, serum should be heated at 56°C/30 min (Heat-inactivated serum) → to destroy non-specific inhibitors in serum which cause false +ve result

• procedure:



25 µL Saline → all wells

25 µL heat-inactivated Serum in first well only → 2 fold serial dilution

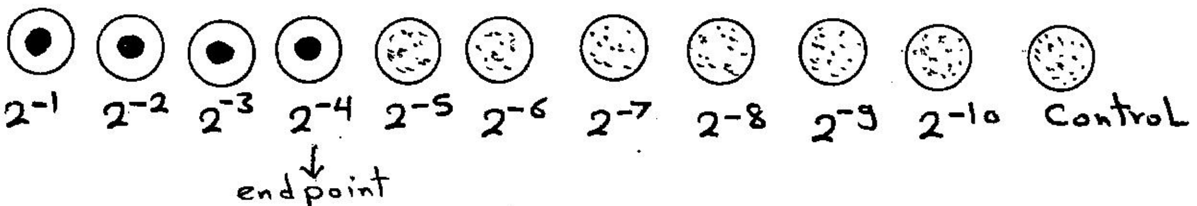
25 µL of virus Contain 4 HAU → all wells

Incubation at 37°C / 1 hr →

25 µL RBCs (0.5 %) → all wells

Incubation at 37°C / 1 hr →

Result:



• **End point** → highest dilution of Abs causing complete inhibition of HA → 2^{-4}

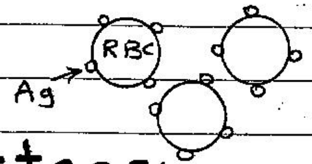
uses of HI test:

1. detection of Ag using Known Abs
2. detection of Abs using Known Ag
3. detection of response to Vaccination (Measurement of immunity)

passive haemagglutination (pHA)
(Indirect HA test = IHA)

→ mainly for Ab detection

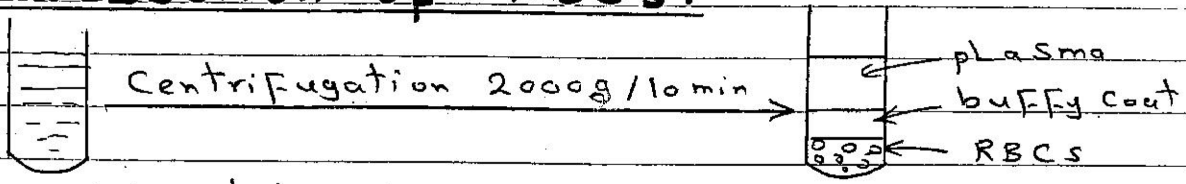
principle:



erythrocyte diagnosticum → RBCs coated by Ag used in pHA for detection of Abs

steps:

1- Collection of RBCs:



heparinized blood of sheep (its RBCs have high adsorptive capacity)

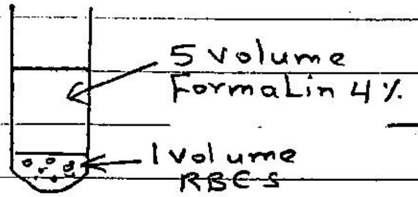
washing 3-4 times by isotonic saline soln (to avoid shrinkage or rupture)

↓

Washed RBCs

2- Fixation of RBCs:

by formalin to ↑ integrity of RBCs cell membrane and ↑ adsorptive capacity of RBCs.

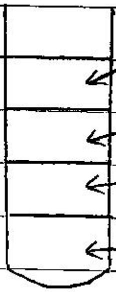


Incubation at 4°c for 3-4 days → then Centrifugation at 2000 g/10 min

↓

Formalin-Fixed Washed RBCs

3- Sensitization of RBCs with antigen:



- 1 volume tannic acid (0.1 - 0.2%) and/or chromium chloride
- 1 volume purified reference Ag
- 1 volume 50% formalin-fixed washed RBCs
- 8 volume saline

↓

incubation at 4°C for 10-15 minutes

↓



centrifugation at 2000 g / 10 min. →



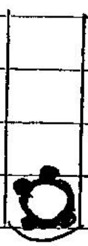
supernatant

RBCs coated with Ag

4- Stabilization of RBCs:

discard supernatant and add equal volume of **30% human serum** (for covering of non-specific sites of RBCs by smooth material) (or **Sucrose** may be used)

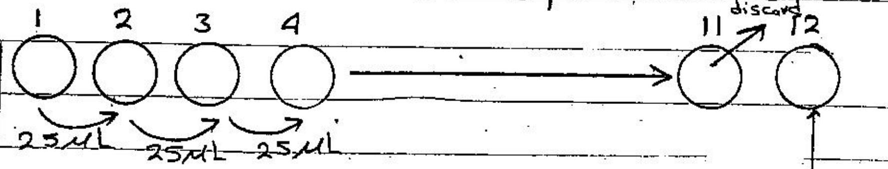
centrifugation 2000 g / 10 min.



erythrocyte diagnosticum

5- testing of unknown antibodies:

- make 2 fold serial dilution of antibodies



① 25 μL Saline

② 25 μL Serum (unknown Ab)

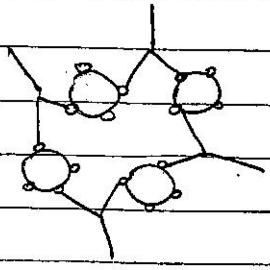
③ Mixing

④ 25 μL erythrocyte diagnosticum 1%

Formalin-fixed non-sensitized RBCs or sensitized with other Ag

⑤ Incubation at room temp/30 min

⑥ Read the results



+ve → Lattice shape

-ve → button - shape

Control → must be button shape

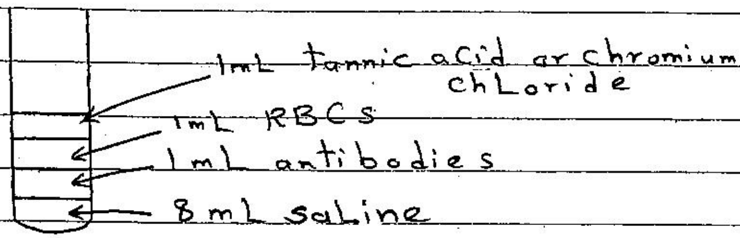
- **Erythrocyte diagnosticums** → erythrocytes sensitized with antigens → prepared mainly from sheep RBCs because they have high adsorptive activity → used in pHA for Abs detection.

Reverse passive haemagglutination (RpHA)

- used for detection of unknown antigen
- Erythrocytes are sensitized by antibodies (Erythrocyte antibody diagnosticums)

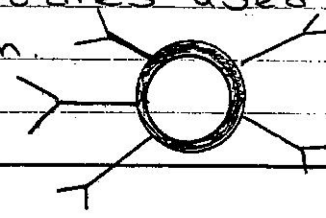
- **steps:**
  1. Collection of RBCs
  2. Fixation of RBCs
  3. Sensitization of RBCs by antibodies
  4. stabilization of RBCs
  5. testing → 2 fold serial dilution of Ag
     → Erythrocyte Ab diagnosticum 1%

Sensitization



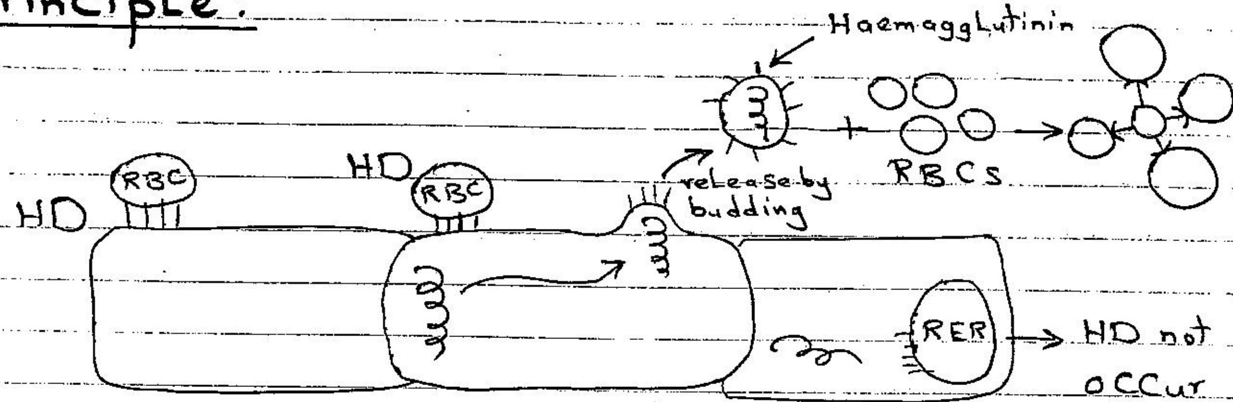
- **Result:**
  - +ve → Lattice shape
  - -ve → button shape

Erythrocyte antibody diagnosticum:
RBCs Coated (sensitized) by antibodies used in RpHA for detection of antigen.



Haemadsorption (HD)

principle:



Virus_infected cells

Haemadsorption (HD):

adsorption of RBCs to virus_infected cells (VIC)

- Cultured cells infected with viruses that bud from the cytoplasmic membrane (such as orthomyxovirus, paramyxovirus and togavirus) have the ability to adsorb RBCs due to incorporation of virus_coded haemagglutinin into the cell's plasma membrane.

- Haemadsorbent virus is haemagglutinating virus but vice versa is not true.

uses of HD:

1. detection of non_cytocidal viruses
2. detection of early stage of infection by cytopathic viruses.

(3) detection of actual time of incorporation of haemagglutinin in cell membrane → give useful information about virus replication.

Viruses cause haemadsorption:

- → orthomyxovirus (Influenza virus)
- → paramyxovirus (NDV)
- → Corona virus
- → Reovirus
- → Togavirus

Factors affecting haemadsorption:

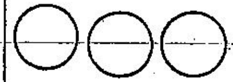
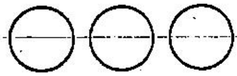
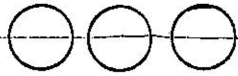
1. Type of cells
2. Type of RBCs and its concentration
3. Type of virus and its concentration
4. Temperature (If neuraminidase is present)
5. presence of non specific inhibitors
6. pH

Steps of Haemadsorption test:

1. preparation of cell culture
2. virus inoculation
3. addition of **0.5% RBCs**
4. examination under inverted microscope

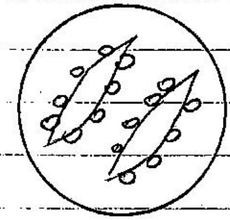
procedure:

Nine petri dishes → divide them into 3 groups (each group Contain 3 dishes)

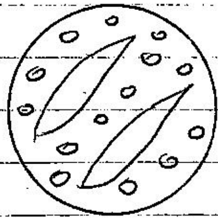
			
1- add 1.5 mL vero cells (african vervet monkey) (1.5×10^5 cells) in all dishes	→	→	→
2- Incubation at 37°C for 24 hrs (for confluent sheet formation)	→	→	→
3- Removal of media	→	→	→
4- virus inoculation (0.2 mL virus per well = 10^{-3} virus)	NDV	Foul pox virus	buffer solution
5- Incubation at 37°C/1h for virus adsorption	→	→	→
6- add 1.5 mL of maintenance media (Eagle's minimum essential medium EMEM)	→	→	→

7- add RBCs (0.5%) in 1st dish of each group **2 hrs** after removal of media	✓ ○ ○	✓ ○ ○	✓ ○ ○
8- Incubation at 37°C for 10 min.	✓ ○ ○	✓ ○ ○	✓ ○ ○
9- Washing by PBS then examination under Inverted microscope	-ve ○ ○	-ve ○ ○	-ve ○ ○
10- after another 2 hrs (**4 hrs**), add RBCs in 2nd dish then incubate, wash and examine under Inverted microscope	○ -ve ○	○ -ve ○	○ -ve ○
11- after another 2 hrs (**6 hrs**), add RBCs to 3rd dish then incubate, wash and examine under Inverted microscope	○ ○ +ve	○ ○ -ve	○ ○ -ve

Results: (under Inverted microscope)



+ve
Haemadsorption



-ve
No haemadsorption

N.B:

HDI → Inhibition of HD by specific antibodies.

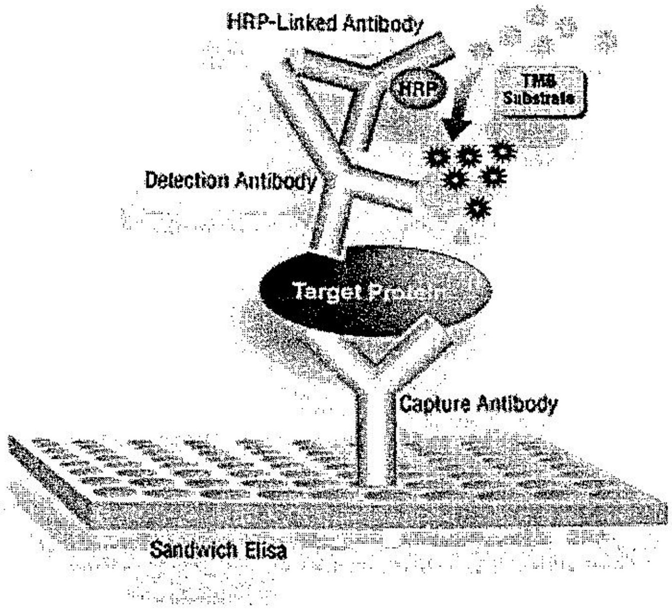
• خلى بالك من تركيز RBCs في كل Test

0.5%	1%	2%	10%
→ HA	→ RpHA	CFT	• Slide (Rapid) HA
→ HI	→ pHA (Indirect HA)		• Tube HA
→ HD			

• **HA**, **HD** — Not serological tests because they do not use specific Abs

Practical Virology
(3)

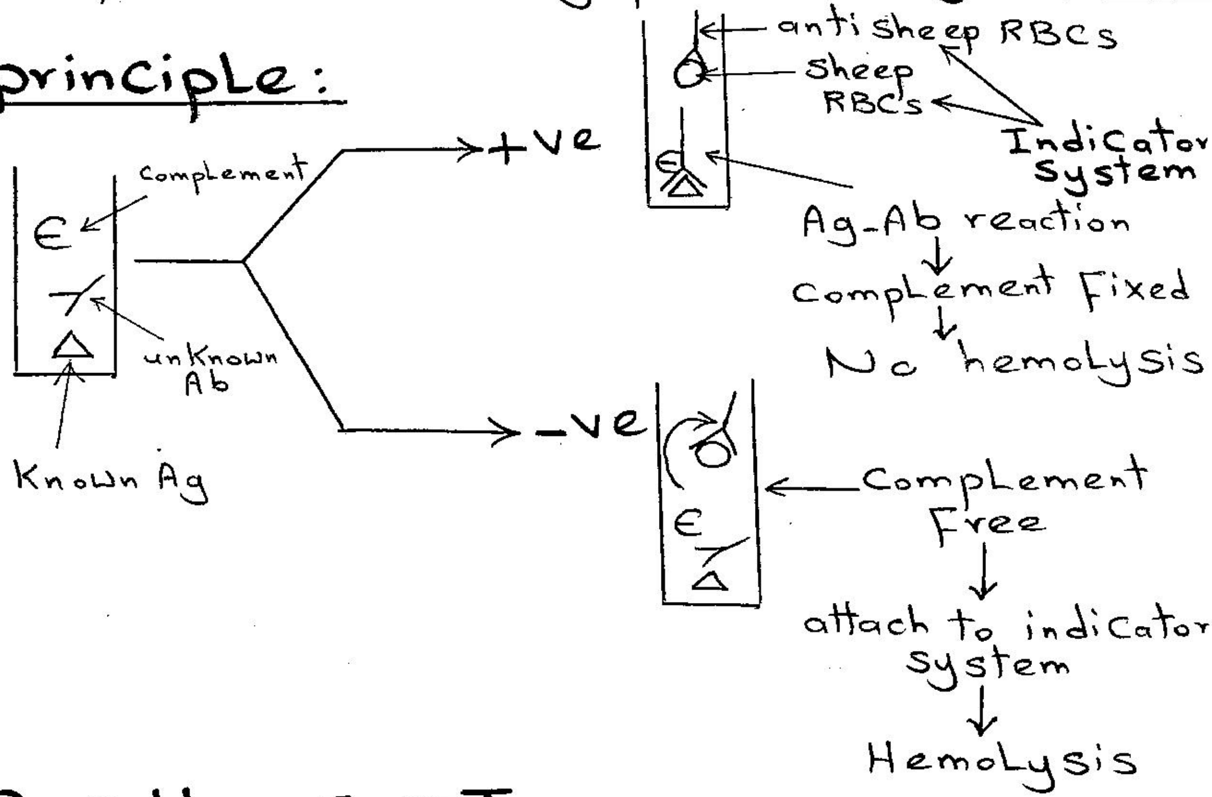
Serological Tests*



Complement Fixation test (CFT)

→ CFT is used mainly for <u>antibody detection</u>.

principle:



Results of CFT:

① +ve result → No haemolysis

② -ve result → haemolysis

N.B:

→ Complement is fixed by Ag-Ab reaction

→ Some antibodies alone Can't destroy Ag untill Complement is fixed.

Reagents:

1. Ag → standard, reference, Known and pure
2. Ab → unknown from patient serum
3. Complement → 2 units

→ **G.pig serum is the source of Complement for CFT because of:**

1- Its high concentration of Complement.
2- its Complement has potent haemolytic activity.

- Ag, Ab and Complement → called **Test or primary system**

4. sheep RBCs 2%

→ **sheep RBCs are used in CFT due to:**

1- have high haemolytic activity (Fragile)
2- More Sensitive to Complement (Complement recepta)

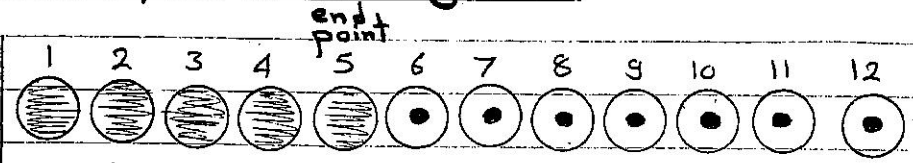
5. antisheep RBCs (haemolysin or Amboreceptors or Incomplete haemolytic system) → at Least 2 units.

- sheep RBCs and antisheep RBCs → called **Indicator system.**

Steps:

1- standarization of haemolysin (2 units)
2- standarization of Complement (2 units)
3- Test → using test tube or microtitre plate with u-shaped bottom.

1- standarization of haemolysin:

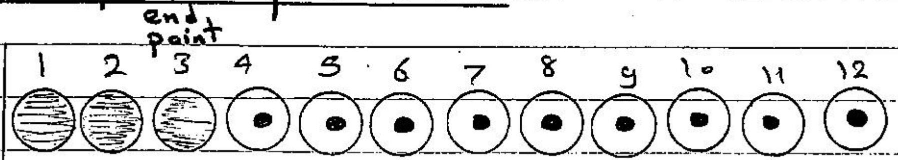


haemolysis No haemolysis (button shape)

① 25 µL saline →
② 25 µL unknown (haemolysin) × 2 fold serial dilution
③ Mixing
④ 25 µL RBCs 2% →
⑤ 25 µL complement →

- **end point** → highest dilution of haemolysin causing complete hemolysis (contain 1 unit of haemolysin) so 2 units of haemolysin من اللى قبلها

2- standarization of complement:

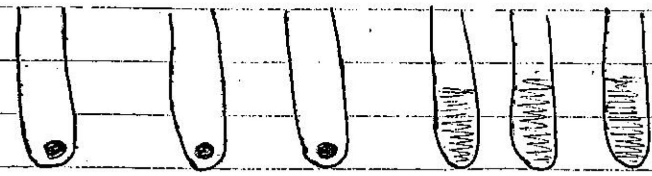


haemolysis No haemolysis

① 25 µL saline →
② 25 µL complement × 2 fold serial dilution
③ 25 µL RBCs 2% →
④ 25 µL anti-sheep RBCs (2 units) →

- **end point** → highest dilution of complement causing complete haemolysis (contain 1 unit of complement) so 2 units of complement من اللى قبلها

3- Test:



Serum dilution	1/2	1/4	1/8	1/16	1/32	1/64
	2^{-1}	2^{-2}	2^{-3}	2^{-4}	2^{-5}	2^{-6}

1. 1/2 mL serum → x 2 Fold serial dilution
2. 1/2 mL Ag →
3. 1mL Complement (2 units) →
4. Incubation at **37°c / 30 min**
5. Sheep RBCs **2%** →
6. anti-sheep RBCs (2 unit) 0.5 mL →

Results:

+ve result → No haemolysis (clear)

-ve result → haemolysis (Turbid)

End point: highest dilution of Ab which cause complete inhibition of haemolysis

advantages of CFT:

1- Rapid

2- Could be done with inactivated virus
3- detection of recent infection

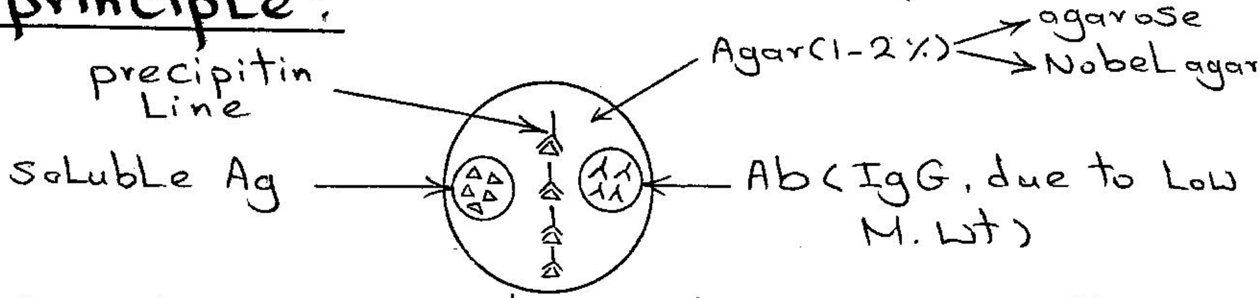
disadvantages of CFT:

1- difficult to be applied
2- Complement fixing antibodies (IgM) persist for short period
3- Some antigens can fix complement without antibodies
4- antibodies must be complement fixing Abs → mainly IgM

dr. M.abdelnaeem
(Bv.sc, Mv.sc, pH.D)
0100 3912 810

Agar gel precipitation test (AGPT) (double immunodiffusion (DID))

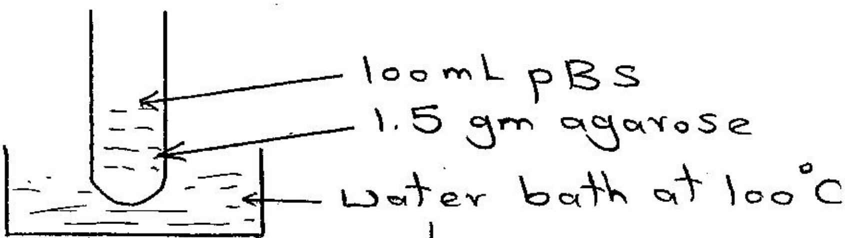
principle:



- Ag and Ab are able to migrate in gel → Ag-Ab complex

+ve result ——→ precipitin Line
-ve result ——→ No precipitin Line

preparation of 1.5 % agarose:



↓

Boiling till dissolving

↓

Cooling to 45 °C

↓

add 0.01 % sodium merthiolate (antifungal)

↓

pouring in petri dish

↓

Incubate at room temp. till solidification

↓

↓

Holes formation by → gel borer
→ gel punch



↓

Sealing of bottom by melted agarose



↓

addition of Ag and Ab

↓

Incubate in humid place at 37°C for 8 hrs – 7 days

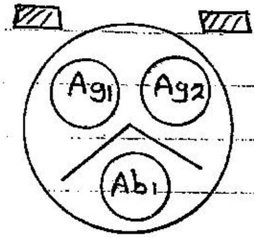
+ve result	-ve result
precipitation Line (Can be stained by commase blue stain)	No precipitation Line

uses of AGpt:

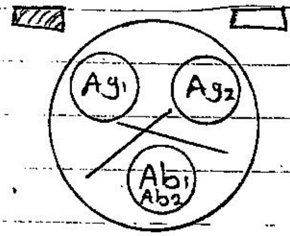
1- detection of Ag or Ab (mainly IgG)
2- determine Concentration of Ag or Ab
3- determine relationship between antigens

use of AGpT for detection of antigenic relationship between 2 antigens:

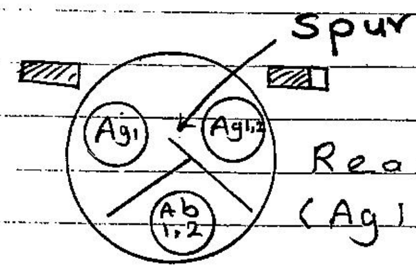
↓



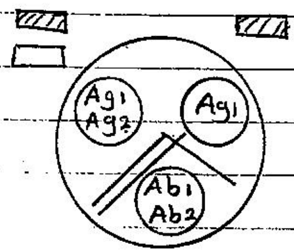
Reaction of identy



Reaction of Non-Identy

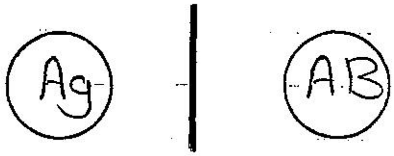


Reaction of partial Identy
(Ag 1,2 on the Same moLecuLe)



Reaction of CompLete identy
and non-identy

N.B:

1- 

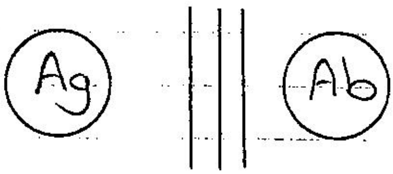
Conc. of Ag = Ab

2- 

Conc. of Ag > Ab

3- 

Conc. of Ag < Ab

4- 

Ag is more than one fraction

• agar of bacteria is not used as:

1- bacteria may grow and interfere with precipitin Line Formation.

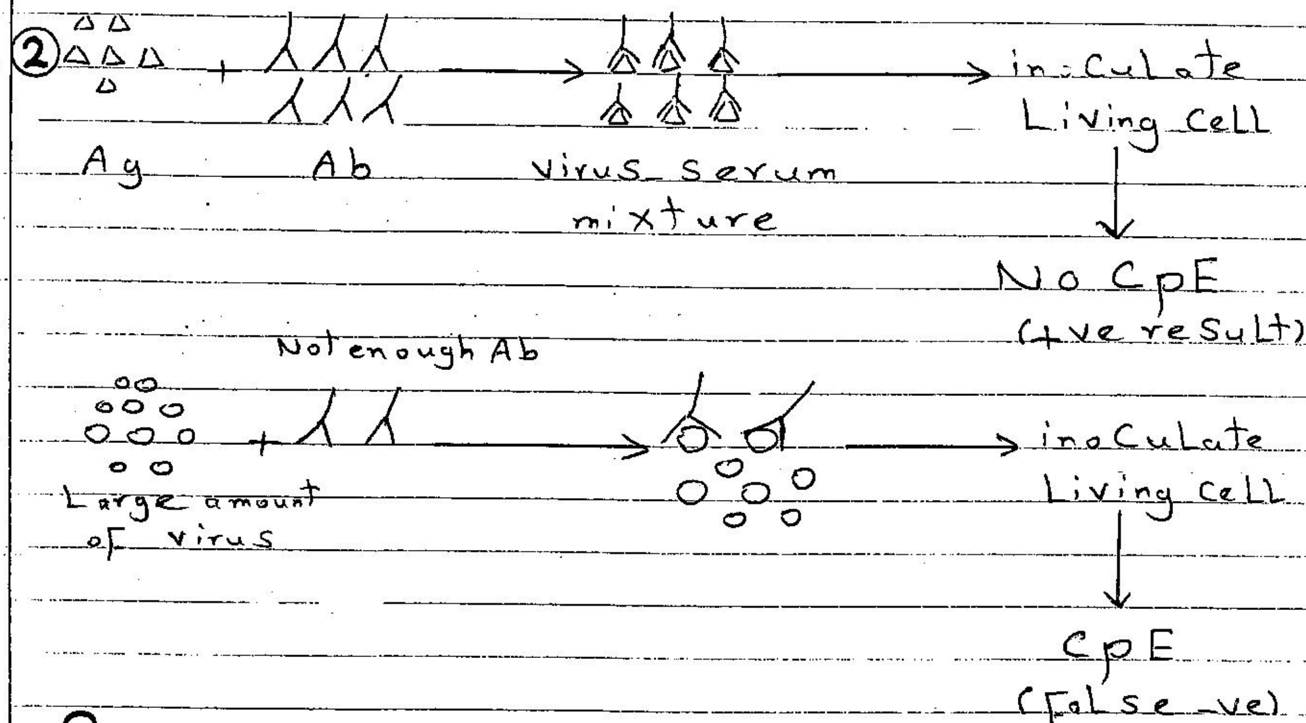
2- this test need agar with high purity
→ So, use agarose or nobel agar.

Neutralization test (NT)
= Serum neutralization test (SNT)

principle:

1. virus → inoculate Living cells
   - → E.C.E → Lesion
   - → TC → CpE
   - → Lab A → death

2.



So... we use:

- Ag → 100 $TCID_{50}$
- Ab → at Least 20 units

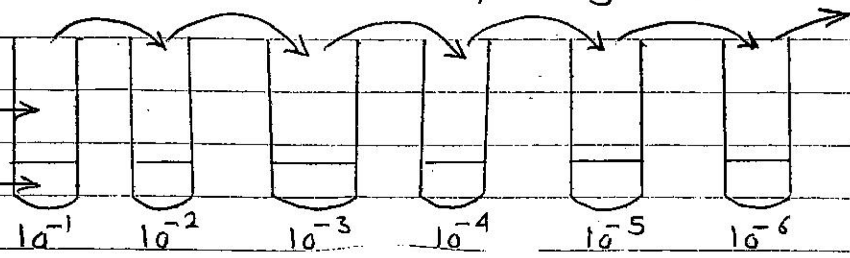
- **$TCID_{50}$** → amount of virus Causing CpE in 50% of inoculated Cell Culture.

1- Titration of virus (100 TCID50)

10 Fold serial dilution of Ag

1mL Virus →

9mL Saline →



① 1 mL Virus — 10 Fold serial dilution of Ag →

② inoculation of 0.2mL in 2 TC

10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
✓ ✓	✓ ✓	✓ ✓	✓ ✗	✗ ✗	✗ ✗

10^{-2} → 100 unit (100 TCID50) المطلوب

10^{-3} → 10 unit

10^{-4} → end point (highest dilution of virus causing CPE in half No. of inoculated cell cultures) contain 1unit (1 $TCID_{50}$)

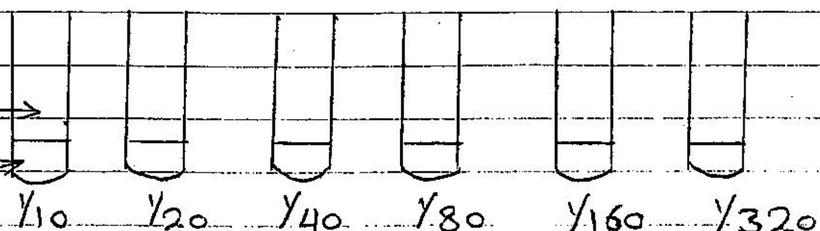
- **Result:**
  ① +ve → CPE
  ② -ve → No CPE

2- Titration of antibody (20 units)

2 Fold serial dilution of Ab

0.5 mL heat-inactivated serum →

0.5 mL saline →



① 0.5 mL Ab — 2 Fold serial dilution of Ab →

② 0.5 mL virus (100 $TCID_{50}$) ⟶

③ Incubation at 37°C / 1 hr ⟶

④ Inoculation in cell culture

1/10	1/20	1/40	1/80	1/160	1/320
✗	✗	✗	✗	✓	✓

↓

End point: end point = 1 unit

→ highest dilution of Ab causing complete inhibition of CpE (complete neutralization to virus)

• Result:

① +ve ⟶ No CpE

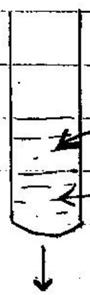
② -ve ⟶ CpE

1/80 ⟶ 1 unit

? ⟶ 20 unit

• dilution contain 20 units ⟶ $\frac{20}{80} = \frac{1}{4}$

3- Neutralization test:



0.2 mL virus (100 TCID50)

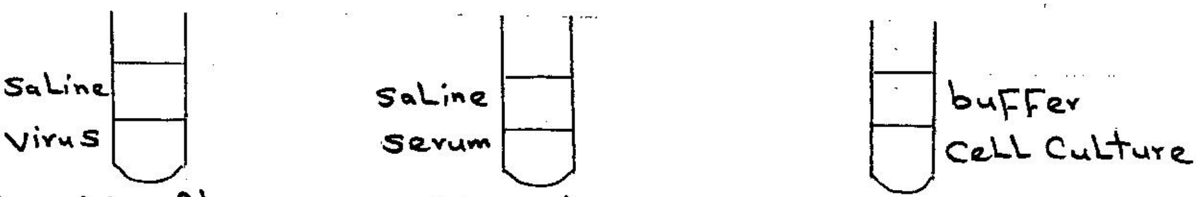
0.2 mL Ab (20 unit)

↓

Incubation at 37°C for 1 hr ⟶ inoculation in cell culture —(incubation for 7 days)→ +ve (No CpE) / -ve (CpE)

N.B:

There should be 3 Control tubes



No Ab — No Virus

Virus Control	Serum Control	Cell Control
−ve	+ve	+ve
↓	↓	↓
CPE	No CPE	No CPE

uses of NT:

1. alpha NT (Varying virus Constant serum)
   ⟶ For detection of virus
2. Beta NT (Constant virus varying serum)
   ⟶ For detection of antibodies
3. detection of serotypes (serotyping) e.g FMDV ⟶ 65 subtypes

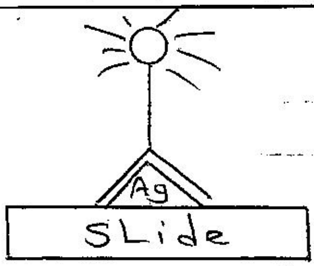
Types of neutralization test:

- → α_method
- → β_method

Immunofluorescence test (IFT)
Fluorescent Antibody technique (FAT)

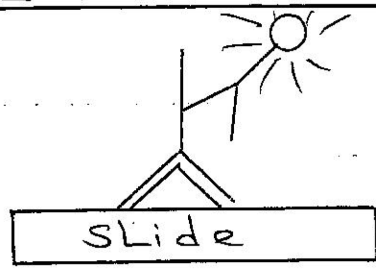
- **principle:**

direct method



Ag + Labeled Ab with Fluorescent dye

Indirect method



Ag + Ab → washing then add antiglobulin Labeled by Fluorescent dye

↓

Washing

↓

examination by Fluorescent microscope (absorption of uv rays by the dye)

+ve

① orange → Rhodamine

② yellowish green → Fluorescein isothiocyanate (FITC)

-ve

• Indicator: Fluorescent dye → absorb UV rays of short wave length (cann't be seen by naked eye) and release them as long wave length (could be seen by naked eye)

- Rhodamine → orange colour
- Fluorescein Isothiocyanate (FITC) → yellowish green (apple green)

• Seen by:

Fluorescent microscope

• uses:

1- detection of virus in T.C

2- detection of site of virus replication

Direct FAT

① Frozen section of tissue (Cryostate)

② Fixation on slide by using **acetone** for 10 min.



③ add Labeled antibody

④ Incubation at **37°c** for **30** minutes at humid place

⑤ washing with 1% PBS

⑥ place the slide in a jar containing PBS for 10 min.

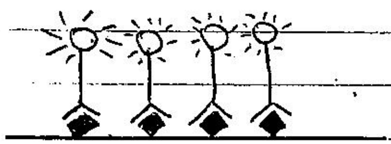
⑦ Remove the slide from the jar and blot dry.

⑧ Cover the stained area with a drop of glycerol and then with a cover slide

⑨ examine under fluorescent microscope with UV source

● **Result:**

① +ve result → yellowish green Colour



② -ve result → No Colour.

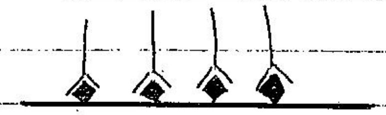
Indirect FAT

① Frozen Section of tissue

② Fixation on slide using acetone



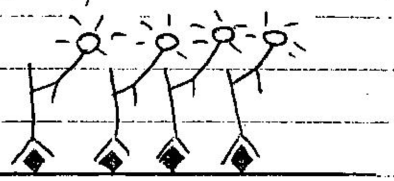
③ add unLabeled Ab



④ Incubation at 37°C for 30 minutes.

⑤ washing by PBS 3 times

⑥ add Labeled antiglobulin (anti-Ab or anti-immunoglobulin or anti-species antibodies)



⑦ Incubation at 37°c / 30 min.

⑧ washing by PBS 3 times

⑨ examination by fluorescent microscope

Result: +ve → Colour; -ve → No Colour

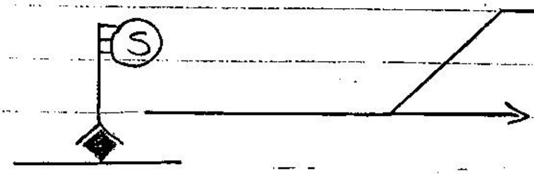
● **Indirect method is better than direct one due to:**

① more sensitive → more than one anti-anti bind to single Ab.

② more convenient → avoid labeling each specific Ab.

EnZyme_LinKed immunoSorbent assay (ELISA)

● principle :



→ +ve change in Colour

→ -ve No Colour

Ag + Ab + E + Substrate

result is seen by Naked eye or ELISA reader or Spectrophotometer

● use microtiter plate with Flat bottom (polystreen)

ELISA

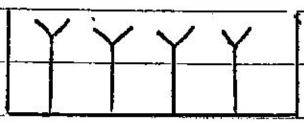
Direct

antibody Capture ELISA (Sandwich ELISA)

● used for detection of unknown antigen

● procedure:

① prepare the Ab coated microtiter plate (Fixation of Ab in plate) (100 μL)



② bLocKing by 1% BSA or Fetal Calf serum, sKimmed miLK, geLatin or Casein

Indirect ELISA (Antigen Capture ELISA)

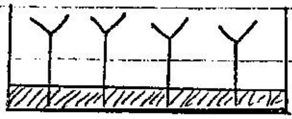
● used for detection of unknown antibody

● procedure:

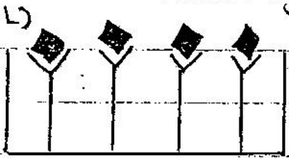
① prepare the Ag coated microtiter plate (Fixation of Ag in plate) (100 μL)



② bLocKing by 1% BSA or Fetal CaLF serum or sKimmed miLK



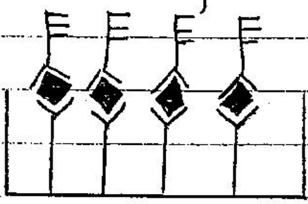
③ add test antigen (100 µL)



④ Incubation at 37°c / 1 hr

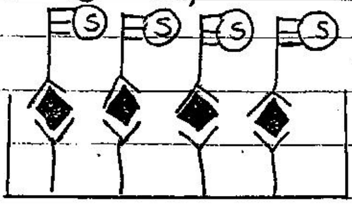
⑤ **washing** by pBS_tween 20 3 times

⑥ add Ab Labeled by enzyme (**Conjugate**) 100 µL → horseraddish peroxidase

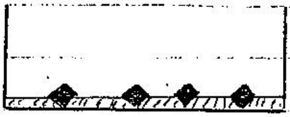


⑦ Incubate at 37°c / 1 hr

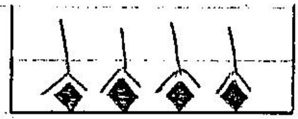
⑧ add **substrate**_indicator solution (ortho-phenylene diamine dihydrochloride_ Hydrogen peroxide) (opD) → Incubate 10 min



⑨ stop the reaction by **stopping soln. (5N H_2SO_4)** because this reaction is **reversible**.



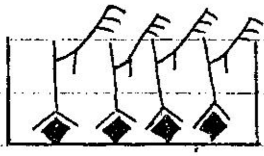
③ add test Ab (100 µL)



④ Incubation at 37c° / 1 hr

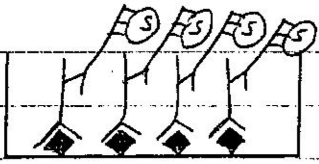
⑤ washing by pBS_tween 20 3 times.

⑥ add anti_Ab Labeled by enzyme (horse raddish peroxidase _anti_immunoglobulin Conjugate) (100 µL)



⑦ Incubate at 37c° / 1 hr

⑧ add substrate_indicator solution → Incubate 10 minutes



⑨ stop the reaction by stopping soln. (5N H_2SO_4)

⑩ Read the result by naked eye or ELISA reader.

Collections

- **serological tests read by naked eye:**
  - → HA
  - → HI
  - → pHA
  - → RpHA
  - → CFT
  - → AGpT
  - → ELISA
  - → Latex agglutination test
  - → NT

- **serological tests not read by naked eye:**
  - → FAT → Fluorescent microscope
  - → NT → Inverted microscope
  - → HD → Inverted microscope
  - → ELISA → ELISA reader
  - → Latex agglutination → Light microscope
  - → Immunoperoxidase → Light microscope

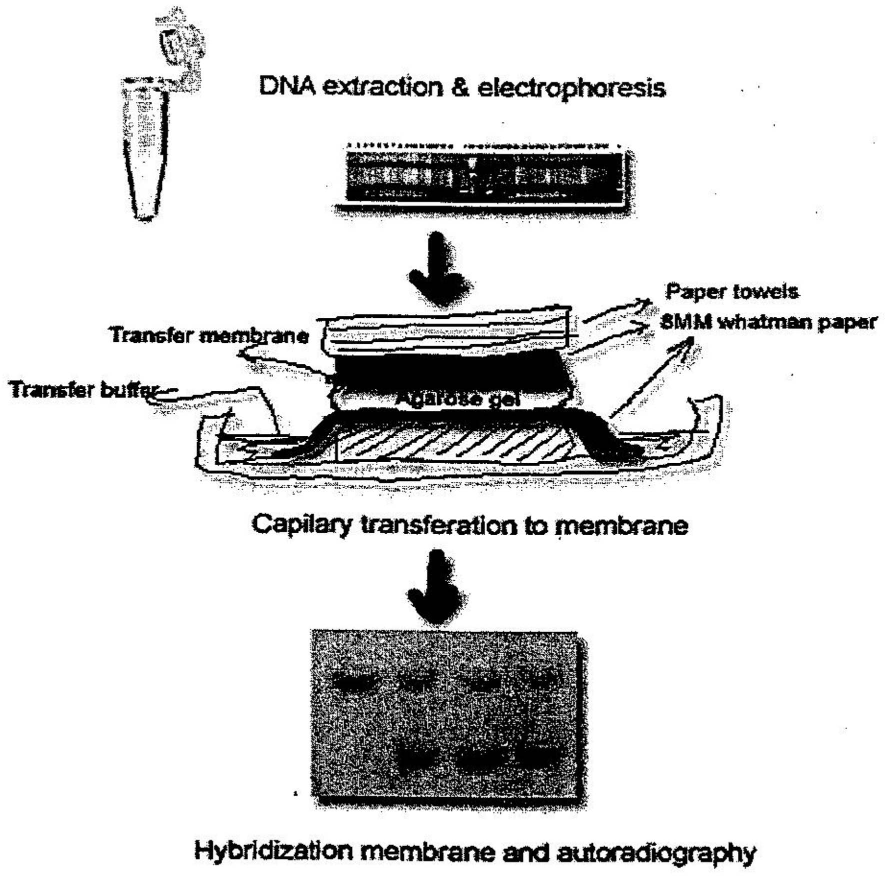
- **detection of non-Cytocidal viruses:**
  - → HA
  - → HD
  - → FAT
  - → EM
  - → Immuno EM
  - → Interference test
  - → Ip

Test	indicator	+ve result	-ve result
1- HA	Washed RBCs 0.5%	haemagglutination (Lattice shape)	No haemagglutination (button shape)
2- HI	Washed RBCs 0.5%	No HA (button shape)	HA (Lattice shape)
3- PHA (Indirect HA)	erythrocyte diagnosticum (1%)	HA (Lattice shape)	No HA (button shape)
4- RPHA	erythrocyte antibody diagnosticum (1%)	HA (Lattice shape)	No HA (button shape)
5- HD	RBCs 0.5%	HD	No HD
6- CFT	Indicator system (sheep RBCs + anti-sheep RBCs)	No hemolysis (clear tube)	Hemolysis occur (turbid tube)
7- AGPT		ppt. Line	No ppt. Line
8- NT (SNT)	Lab. animal, TC, ECE	No CPE	CPE
9- FAT (IFT)	Fluorescent dye (Rhodamine or Fluorescein isothiocyanate)	orange or yellowish-green colour	No colour
10- ELISA	Substrate (ortho-phenylene-diamine dihydrochloride-H_2O_2) (OPD)	Colour change seen by naked eye or ELISA reader.	No colour

Practical Virology

(4)

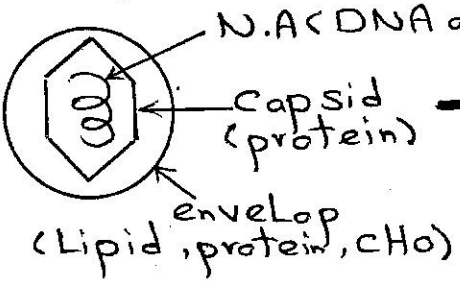
Molecular Techniques



Molecular Techniques

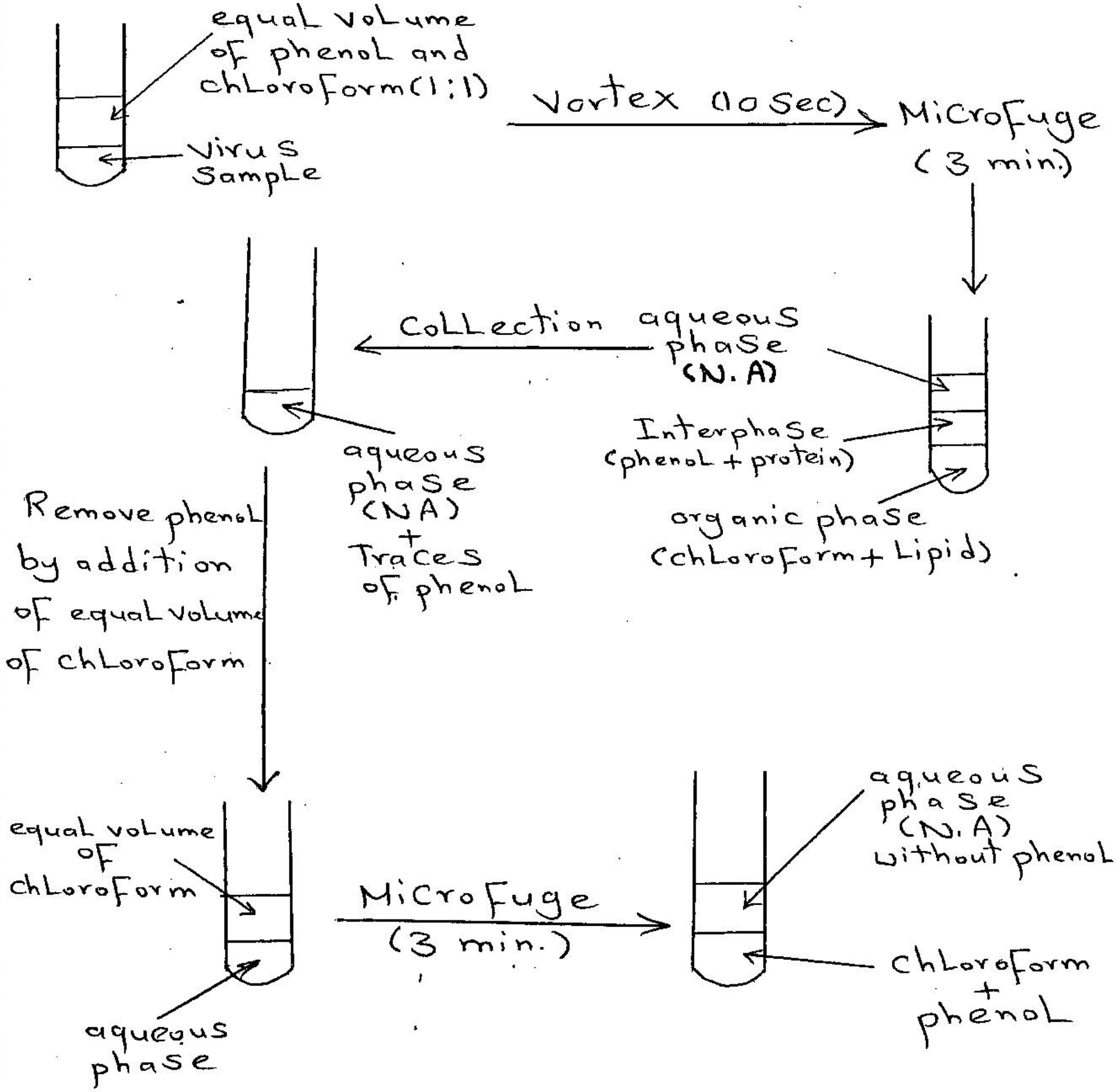
def. detection (identification) of viral nucleic acid.

steps:



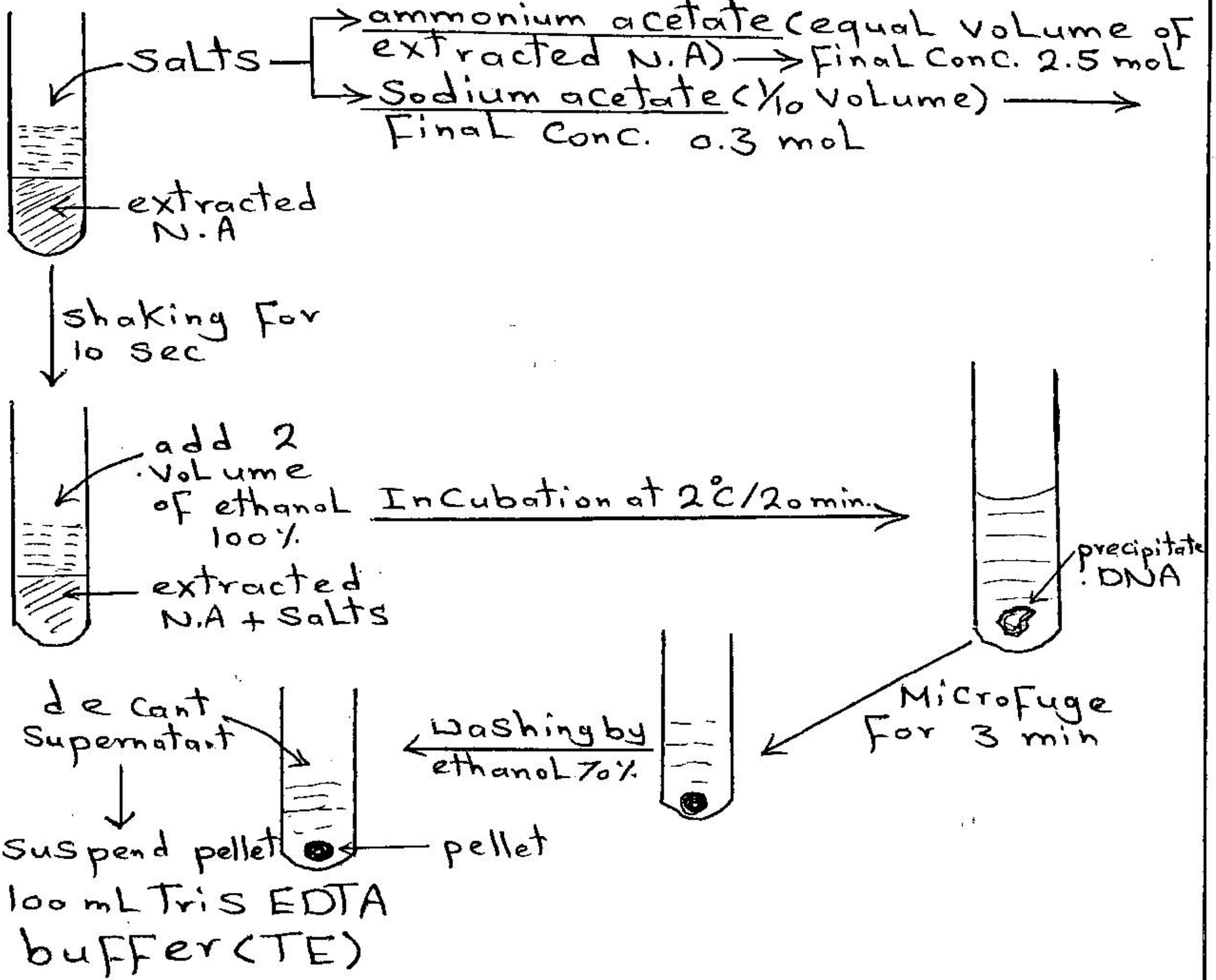
1. Extraction of N.A by phenol and chloroform
2. precipitation of DNA by salts and ethanol
3. amplification of viral nucleic acid
   - In vivo
     - DNA cloning
   - In vitro
     - DNA → PCR
     - RNA → RT-PCR
4. Restriction endonuclease assay
5. Identification of amplified nucleic acid
   - gel electrophoresis (agarose, poly-acrylamide)
   - Hybridization
     - Southern blotting
     - Northen blotting
     - Dot-blot
     - In-situ
   - N.A Sequencing
     - Sanger (chain termination)
     - Maxam Gilbert (chemical) method

(1) Extraction of nucleic acid (DNA) by phenol and chloroform:



② precipitation of DNA:

by using → ethanol 100%
→ Salts → Sodium acetate (3 mol)
→ ammonium acetate (5 mol)

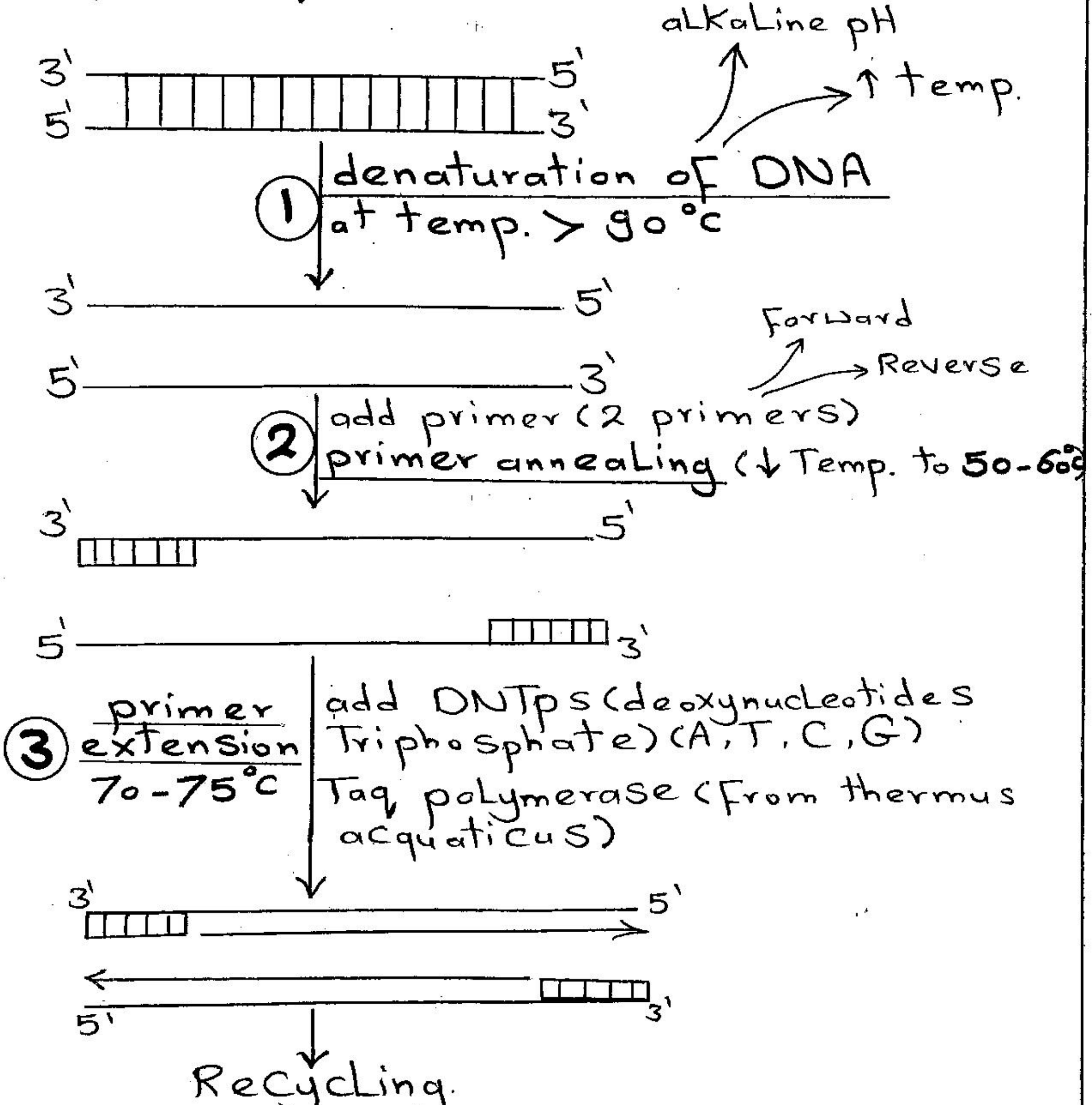


---

③ amplification of DNA by polymerase Chain reaction (PCR):

def. In-vitro enzymatic amplification of DNA

principle of PCR :



primer : short Known Sequence of DNA (18 - 24 bases) Complementary to 3' end of each strand.

steps of pCR :

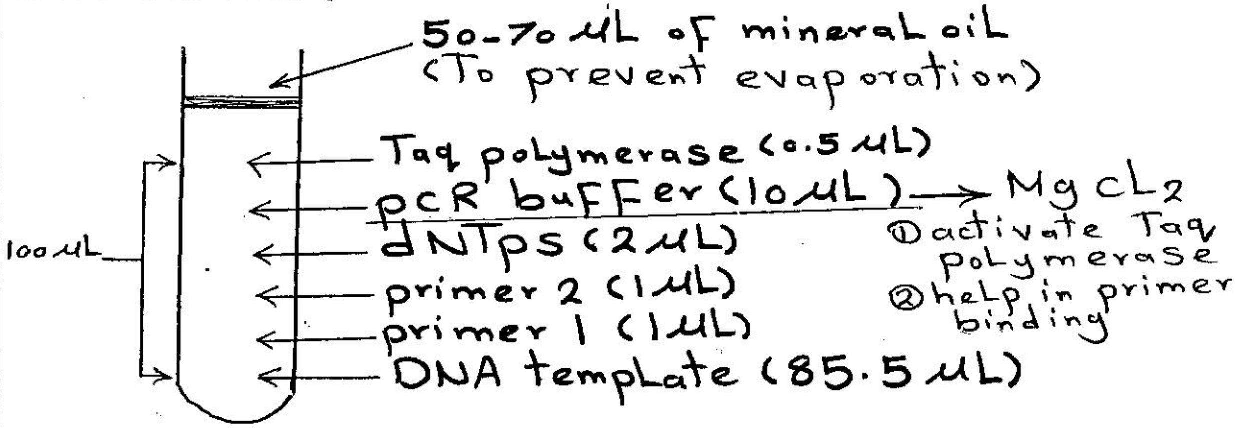
1. denaturation ⟶ > 90 °C
2. primer annealing ⟶ 50 – 60 °C
3. primer extension ⟶ 70 – 75 °C
4. Recycling

$$N = 2^n$$

n ⟵ No. of Cycles

N ⟶ No. of amplified DNA (pCR product)

Method :



↓

Thermocycler

programming:

1- 95 °C ⟶ 1 min
2- 55 °C ⟶ 1 min
3- 72 °C ⟶ 1 min (< 500 bp)
           ⟶ 3 min (> 500 bp)

No. of Cycles ⟶ 30 – 40 Cycles

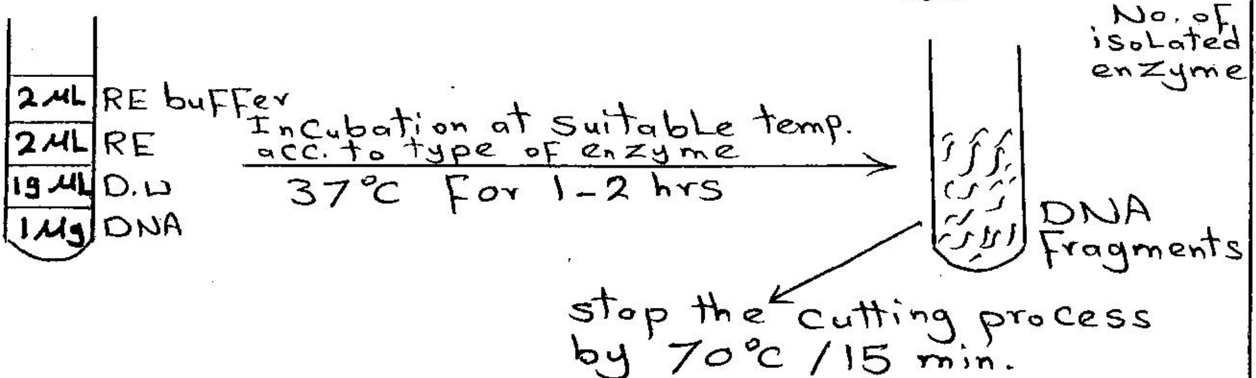
Final extension ⟶ 72 °C / 10 min.

N.B: pCR is not a diagnostic test but it is used for amplification of DNA.

(4) Restriction endonuclease assay (REA):

- Restriction endonuclease → Cutting of DNA at specific sequence (DNA scissors) → It is isolated from bacteria e.g ECoRI

E → Echirchia, Co → CoLi, R → restriction, I → No. of isolated enzyme

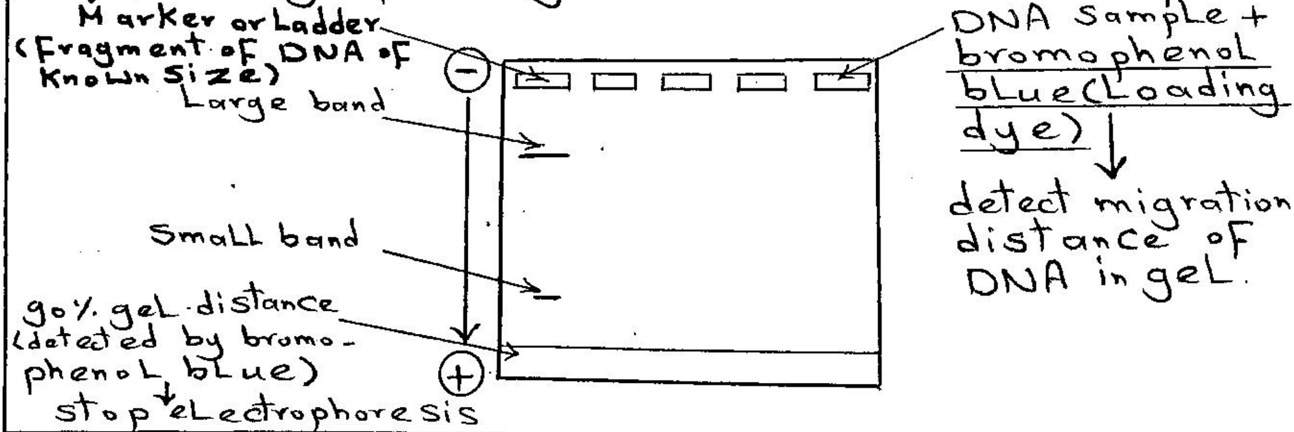


(5) detection of amplified products:

gel electrophoresis | hybridization | N.A sequencing

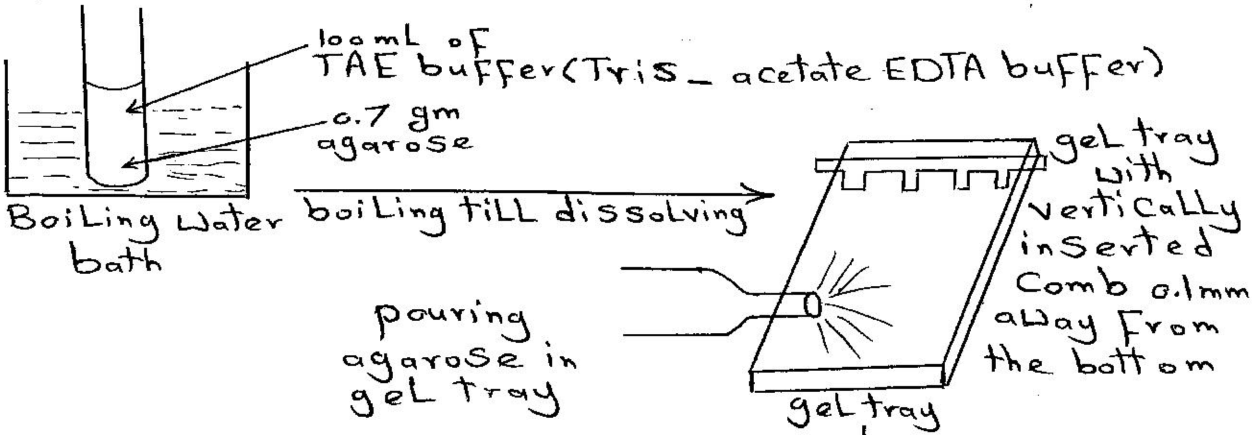
gel electrophoresis

def. Moving of charged molecule in electric field.



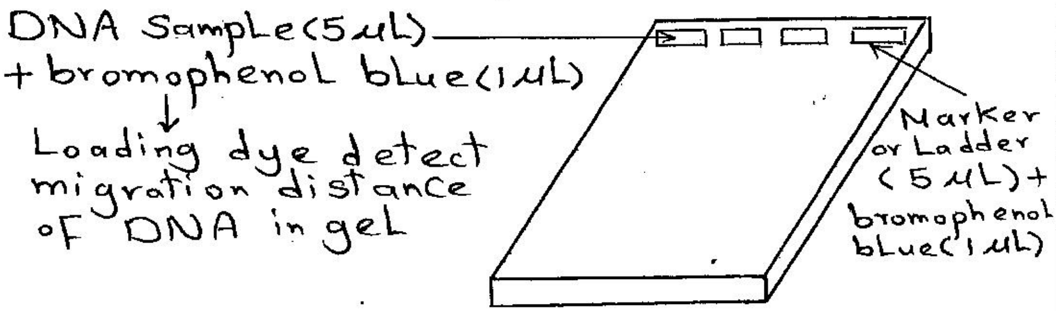
steps:

1- preparation of agarose gel 0.7 %



Incubation at room Temp. / 20 min. till solidification then remove Comb

2- Loading of DNA Sample in gel:



3- electrophoresis at 50 _ 150 volts:

stop electrophoresis when Loading dye migrate 90% gel distance.

4- Visualization of DNA in gel:

staining by Ethidium bromide (0.5 uL/mL for 10 min.) in staining jar.

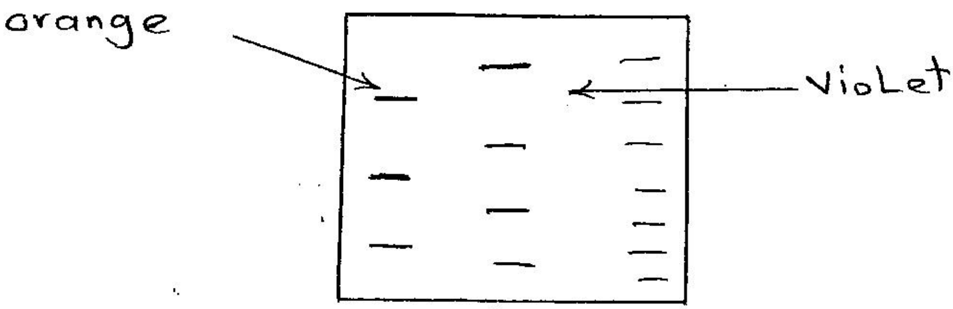
↓

Washing by D.W in washing jar

↓

Examination under UV rays by ultraviolet Transillumination

↓



↓

Take image by polaroid Camera

↓

black gel with white DNA bands

N.B:

Visualization of DNA in gel:

1- staining by ethidium bromide (Et.Br) → which intercalated between double strands of DNA (used only for dsDNA) → examination under UV rays to see the dye → orange Colour.

2- autoradiograph.

Questions:

1- DNA extracted by phenol and chloroform.

2- precipitated by salts and ethanol 100%

salts → ammonium acetate (5 mol)

salts → Sodium acetate (3 mol)

3- amplification of N.A by

→ In vivo cloning

→ In vitro → DNA → pcR

→ In vitro → RNA → RT_pcR

4- gel for DNA electrophoresis consists of 0.7 gm agarose + 100 mL TAE buffer

5- DNA migrate in gel after mixing with Bromophenol blue (Loading dye detect migration distance of DNA in gel)

6- electrophoresis at 50_150 volts and visualization of DNA in gel by:

1_ staining by Ethidium bromide (intercalated in ds of DNA)

2_ autoradiograph

7- Migration of DNA in gel depend on charge and size

→ Small fragments migrate faster than Large one

HybridiZation (probe TechnoLogy)

principLe:

DNA

denaturation:
1- ↑ Temp.
2- aLKaLine pH

add LabeLLed probe (SSDNA of Known organism ComplementAry to ViraL DNA and must be LabeLLed)

LabeLLing probe

- radioactive substance (p^{32}) → detected by autoradiograph
- Non-radioactive substance
  - Biotin-Avidin Reporter → CoLorimetric detection
  - Chemi-Luminescent reporter → detection of emitted Light

+ve resuLt → dupLex Formation

-ve resuLt → No dupLex Formation

Types of hybridization

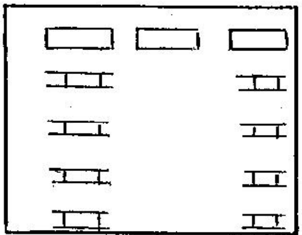
- Liquid phase
- SoLid phase
  - Southern bLotting (DNA)
  - Northen bLotting (RNA)
  - dot-bLot (DNA + RNA)
- In Situ

Northen (RNA)

Western (protein) ↔ Eastern (protein post-translation modification)

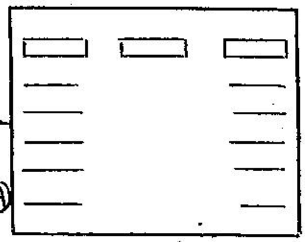
Southern (DNA)

Southern bLotting hybridization



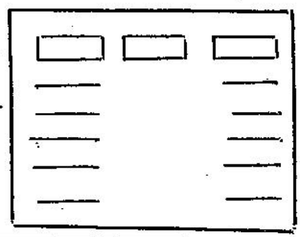
geL eLectrophoresis

→ denaturation by aLKaLine pH (NaoH) For 25 min. (to obtain SSDNA) →



SingLe-stranded DNA

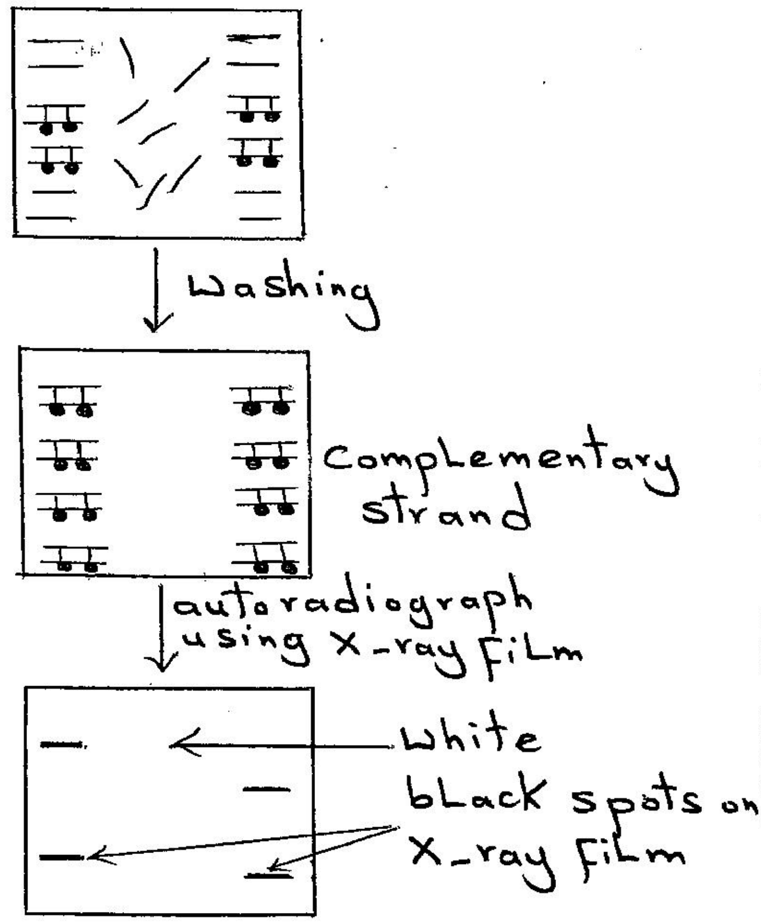
↓ BLotting (Transfer of DNA from geL to nyLon or nitro CeLLuLose membrane)



← Fixation of DNA on nyLon membrane by:
① uLtravioLet Cross Linker
② InCubation at 80°c/2 hrs

addition of probe (Hybridization)

InCubation at 37°C/30 min. ↓



steps of southern blotting:

1. Restriction endonuclease enzyme digestion
2. gel electrophoresis
3. denaturation
4. blotting
5. Fixation
6. probe addition ⟶ hybridization
7. Visualization ⟶ autoradiograph

Blotting:

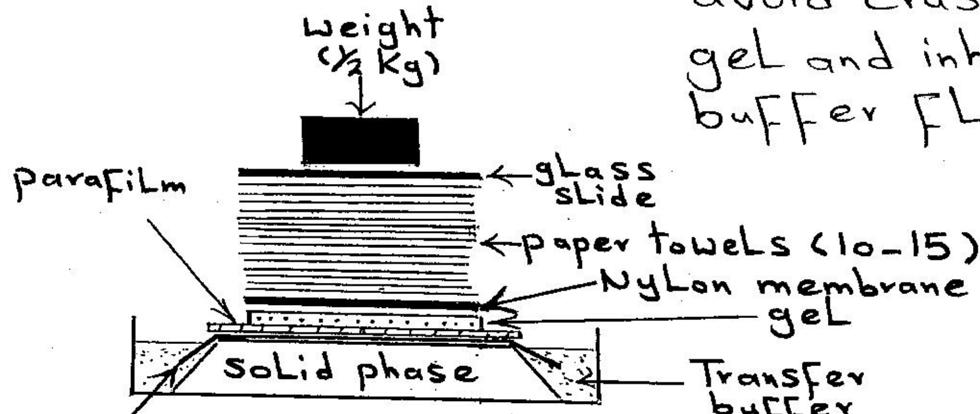
transfer of DNA from gel to nylon or nitrocellulose membrane

upward capillary transfer

more common

downward capillary transfer

more preferable to avoid crushing of gel and inhibition of buffer flow.



(saline sod. citrate)
(SSC)

blotting paper (the end of filter must be embedded in transfer buffer)

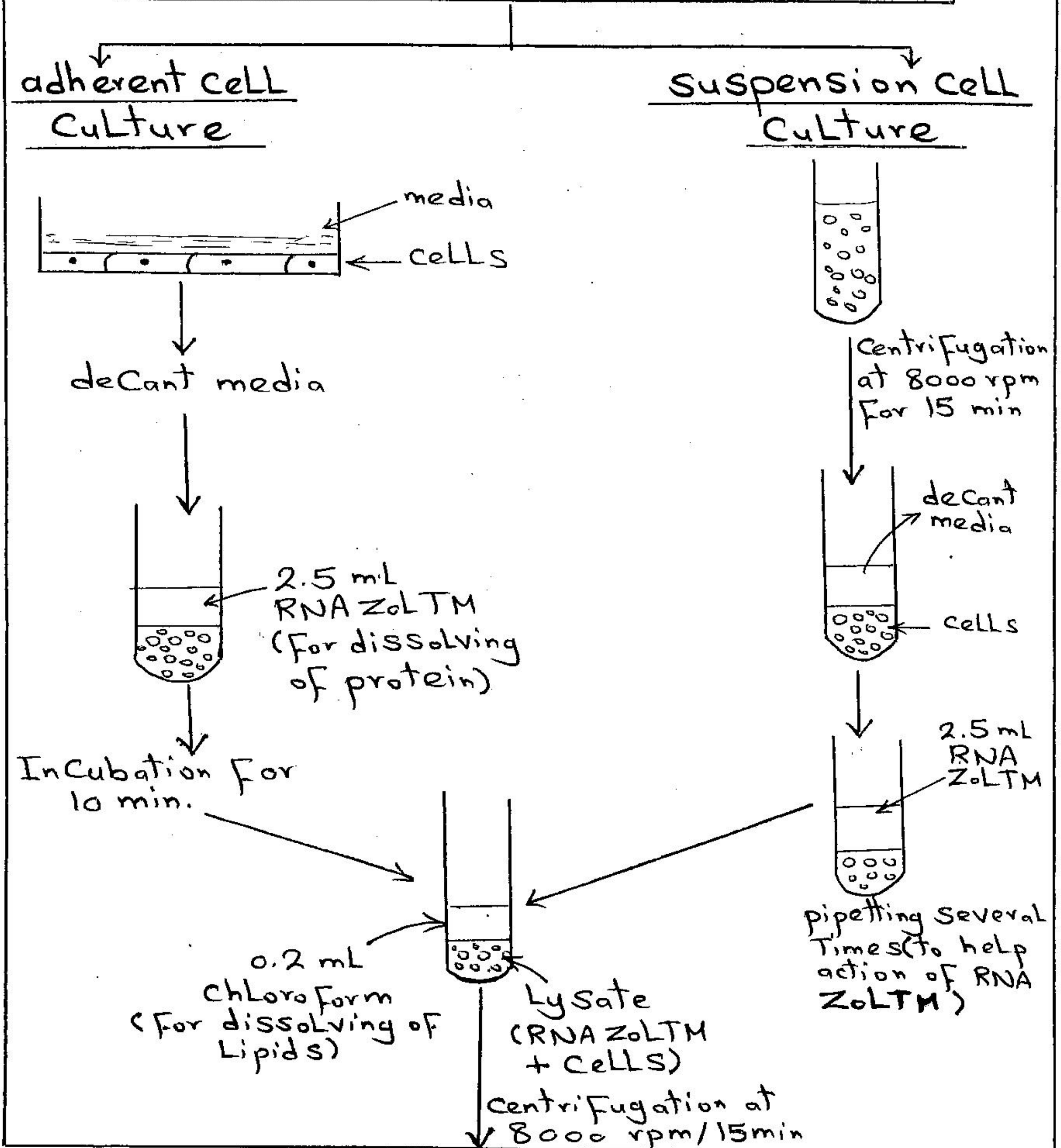
Incubation at room temp. for 24 hrs.

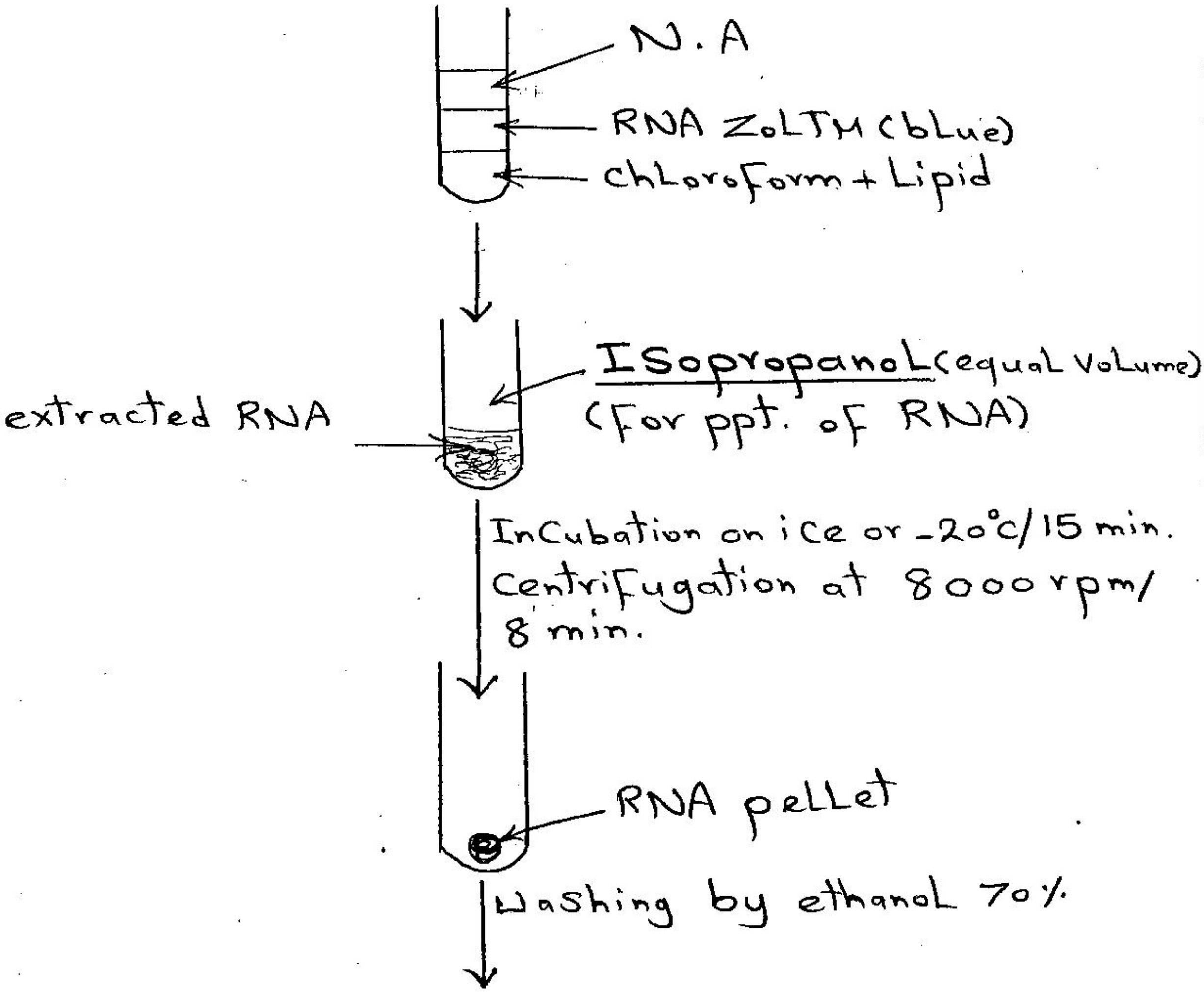
↓

N.A in nylon membrane (+ve)

upward capillary Transfer

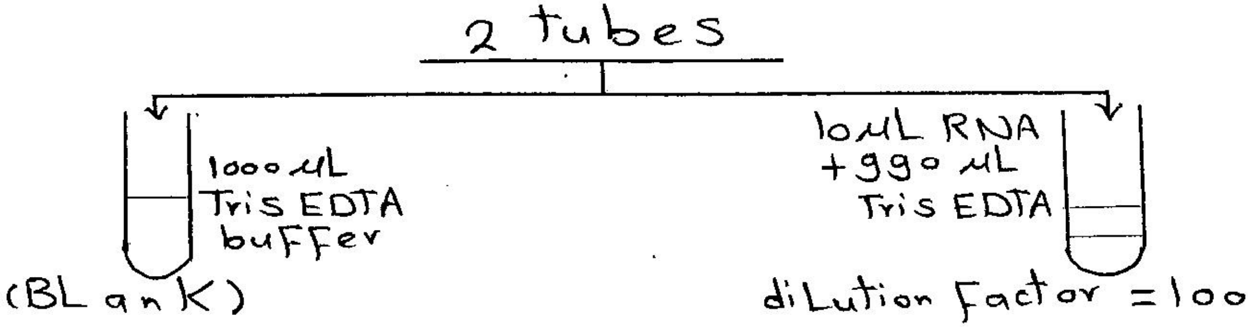
Extraction of RNA by RNA ZoLTM and chLoroform





dissolve in 200 µL Tris EDTA buffer

Concentration of RNA:



detect optical density by Spectrophotometer

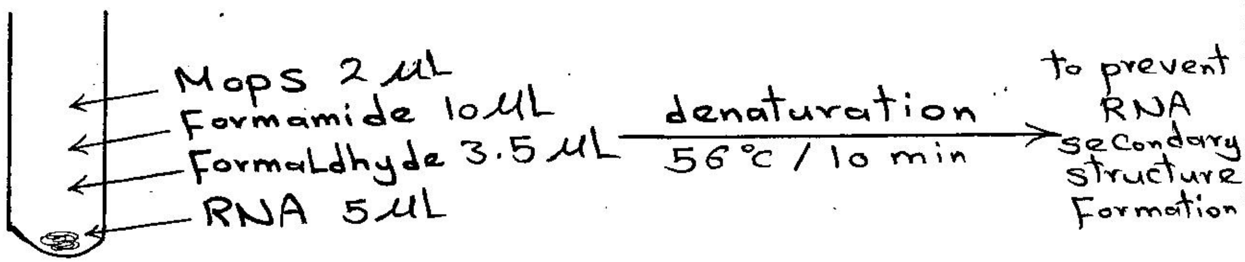
Conc. = optical density (o.D) × dilution factor × Conversion factor → 50 (DNA) → 40 (RNA)

Conc. = o.D × 100 × 40 = μg/mL

Northen blotting hybridization

→ used for detection of RNA.
→ RNA is single-stranded but RNA has ability to make intermolecular hydrogen boat leading to RNA secondary structure formation → so make denaturation.

1- denaturation of RNA → by heat



- Formamide and Formaldhyde → to prevent RNA secondary structure formation (maintain RNA as single strand)
- Mops (Morphlinoproban sulphuric acid) → Inactivate RNase

2- denaturating geL :

Composed of

- 115 mL D.W
- 15 mL Formamide
- 0.5 gm agarose

→ Boiling 100°C → Cooling to 45 °C → add 24.5 mL Formaldhyde → pour in gel Tray

add 24.5 mL Formaldhyde → To prevent RNA secondary structure Formation

---

→ denaturation at 56°C / 10 min → gel electrophoresis in denaturating geL → BLotting → hybridization → Washing → autoradiograph

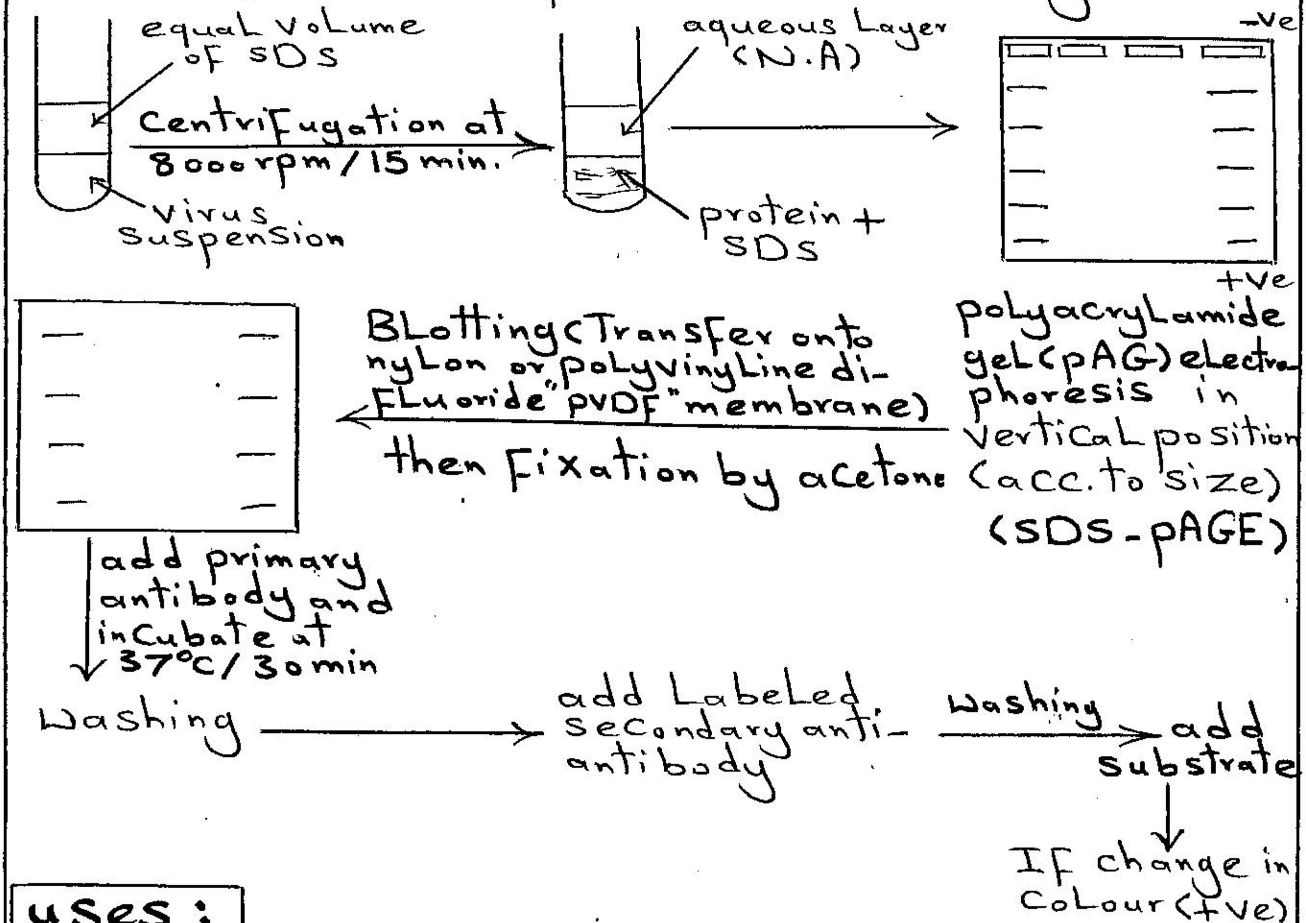
---

steps of Northen bLotting :

1. RNA denaturation.
2. geL electrophoresis
3. BLotting
4. hybridization
5. autoradiograph

Western bLotting technique

- used for detection of protein.
- Extraction of protein by SDS (Sodium dodecyL sulphate) which cover protein by -ve charge so, migration depend on size onLy.



uses:

1. detect serum antiviraL Abs to specific viraL protein
2. Confirm doubtfuL resuLts obtained by seroLogicaL tests.
3. detect viraL Ag in cLinicaL specimen.

points of difference	Southern bLotting Technique	Northen bLotting Technique	Western bLotting Technique
detect	DNA	RNA	protein
extraction	phenoL-chLoroform	RNA ZoLTM-chLoroform	Sodium dodecyL suLphate(SDS)
geL	agarose geL 0.7%	agarose + Formamide + Formaldhyde (denaturating geL)	poLyacryLamide geL (pAG)
Membrane	NyLon	NyLon	NyLon or poLyvinyLine DiFLuoride (pVDF)
Indicator	LabeLed probe	LabeLed probe	LabeLed antibodies by enzyme
+ve ResuLt	autoradiograph	autoradiograph	change in CoLour

Practical Virology

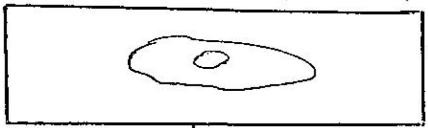
(5)

Molecular Techniques



In situ hybridization

- used for detection of <u>DNA or RNA inside tissues.</u>



Frozen tissue section

↓

Fixation by paraformaldehyde 4% / 10 min.
(maintain cell details)

↓

proteinase_K enzyme / 10 min. which
→ ↑ permeability of cells to probe
→ dissolve protein bound to nucleic acid

↓

prehybridization (optional)
by H_2O_2 (block enzymatic activity of the cell)

↓ denaturation
1- DNA → by alkaline soln.
2- RNA → incubation at 56°C / 10 min.

↓

Single stranded

↓

addition of labeled probe (by radioactive sub.)

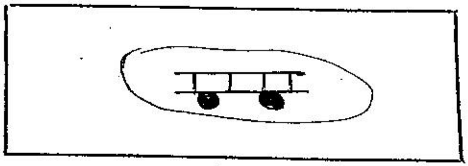
↓

Incubation at 55°C overnight

↓

washing

↓



↓ add nuclear emulsion (Kodak) at 42 °C / 2 hrs

Immersion in developer (15°C / 3 min.)

↓

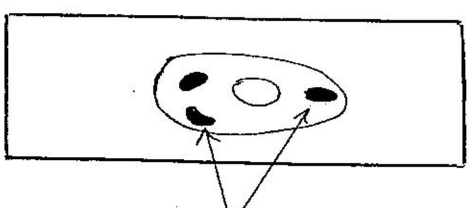
Immersion in Fixer (15°C / 3 min.)

↓

stain by H/E as Counter stain

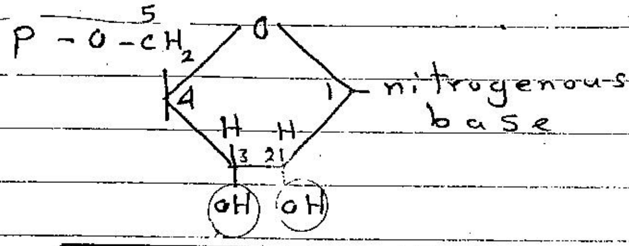
↓

Examine under Light microscope



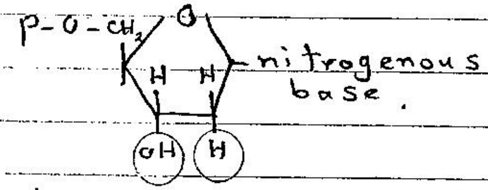
DNA Sequencing

- Sanger or dideoxy or chain termination method
- Maxam Gilbert or Chemical method

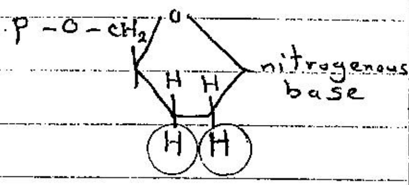


Ribonucleotide

− O | − O_2



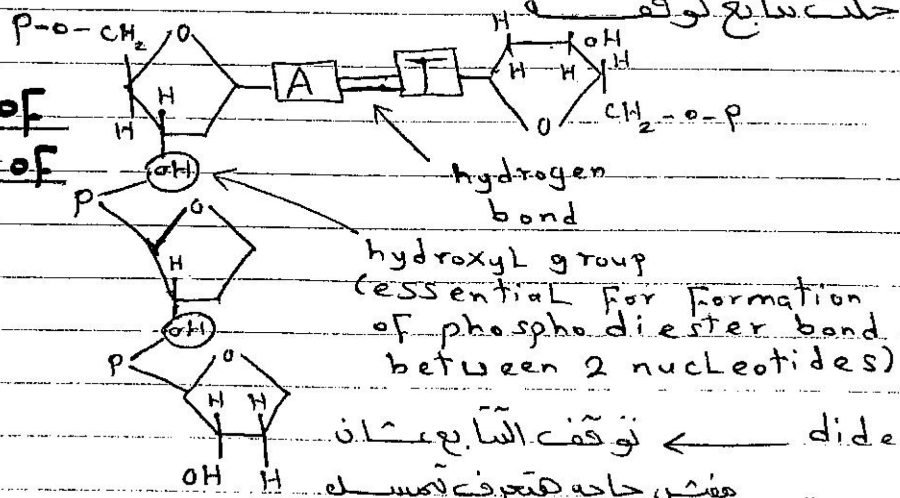
deoxyribonucleotide



dideoxy ribonucleotide

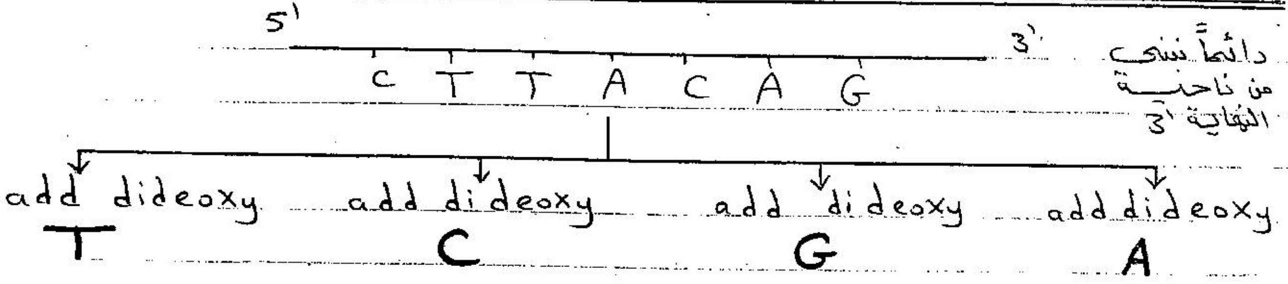
لو دخلت تتابع توقفه

structure of nucleotide of DNA:



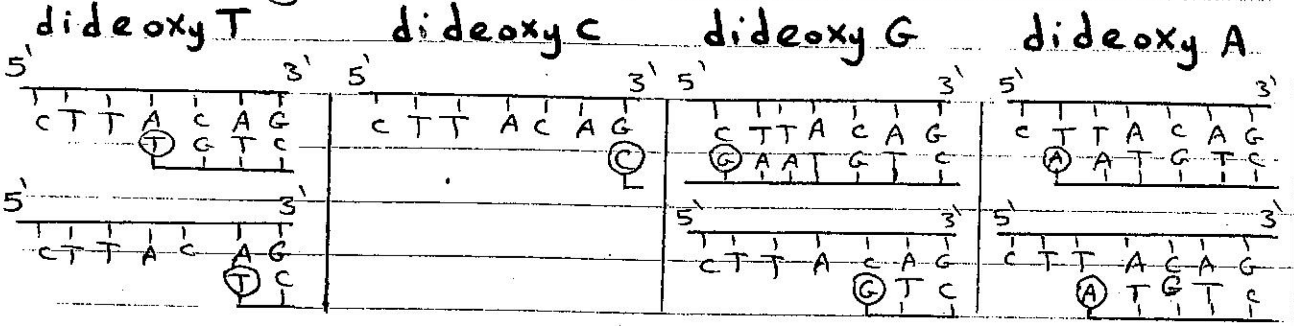
- dideoxy ← توقف التتابع عشان مفيش حاجه هتعرف تمسك فيها

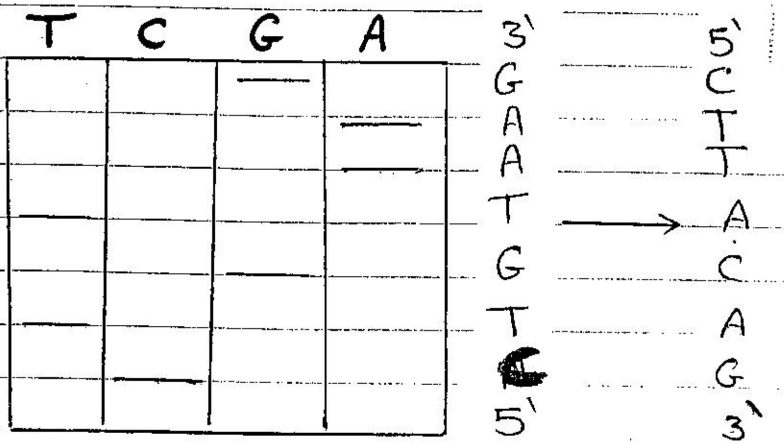
1- Sanger or dideoxy or chain termination method:



دائماً نبنى من ناحيــة النهاية 3'

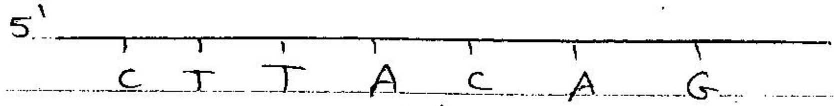
1. DNA templete
2. Labeled primer
3. dNTps (A, G, C, T)
4. Taq polymerase
5. dideoxy





- visualization of band by → autoradiograph
  → Fluorescence

2- Maxam Gilbert (chemical) method:



البدايه 3'
دائما من عند
النهايه 3'

by using chemicals that cut the DNA at specific nucleotides

Cut at G | Cut at A and G | Cut at C and T | Cut at C

